AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

HOST RESPONSE AND INFLAMMATION

Participation of the Serine Protease Jonah66Ci in the *Drosophila* Antinematode Immune Response

Shruti Yadav,[a] Ioannis Eleftherianos[a]

[a]Infection and Innate Immunity Lab, Department of Biological Sciences, The George Washington University, Washington, DC, USA

ABSTRACT Serine proteases and serine protease homologs form the second largest gene family in the *Drosophila melanogaster* genome. Certain genes in the *Jonah* multigene family encoding serine proteases have been implicated in the fly antiviral immune response. Here, we report the involvement of *Jonah66Ci* in the *Drosophila* immune defense against *Steinernema carpocapsae* nematode infection. We find that *Drosophila Jonah66Ci* is upregulated in response to symbiotic (carrying the mutualistic bacterium *Xenorhabdus nematophila*) or axenic (lacking *Xenorhabdus*) *Steinernema* nematodes and is expressed exclusively in the gut of *Drosophila* larvae. Inactivation of *Jonah66Ci* provides a survival advantage to larvae against axenic nematodes and results in differential expression of Toll and Imd pathway effector genes, specifically in the gut. Also, inactivation of *Jonah66Ci* increases the numbers of enteroendocrine and mitotic cells in the gut of uninfected larvae, and infection with *Steinernema* nematodes reduces their numbers, whereas the numbers of intestinal stem cells are unaffected by nematode infection. *Jonah66Ci* knockdown further reduces nitric oxide levels in response to infection with symbiotic *Steinernema* nematodes. Finally, we show that *Jonah66Ci* knockdown does not alter the feeding rates of uninfected *Drosophila* larvae; however, infection with axenic *Steinernema* nematodes lowers larval feeding. In conclusion, we report that *Jonah66Ci* participates in maintaining homeostasis of certain physiological processes in *Drosophila* larvae in the context of *Steinernema* nematode infection. Similar findings will take us a step further toward understanding the molecular and physiological mechanisms that take place during parasitic nematode infection in insects.

KEYWORDS *Drosophila*, *Steinernema*, innate immunity, parasitism, proteases

Drosophila melanogaster is an established model for dissecting the molecular and cellular basis of host-pathogen interactions (1). Extensive studies have led to the identification and understanding of evolutionarily conserved signaling pathways that are activated in response to different types of microbial infections (2–4). *Drosophila* has been employed recently to dissect the molecular mechanisms that occur in insects responding to parasitic nematode infections (5–8). The *Drosophila* immune system shares significant homology to the mammalian innate immune system, which facilitates modeling parasitic processes and antinematode immune reactions in humans (9–11).

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are emerging as excellent models for studying insect-nematode interactions (9, 11, 12). They are natural obligate parasites of a wide range of insects that they infect to complete their life cycle. These nematode parasites infect susceptible insects as infective juveniles, a developmentally arrested stage analogous to the *Caenorhabditis elegans* dauer stage (12). A distinct feature of entomopathogenic nematodes is the presence of mutualistic bacteria that are localized to their intestines (13, 14). *Steinernema carpocapsae* forms a mutualistic relationship with the Gram-negative bacterium *Xenorhabdus nematophila* (symbiotic nematodes), and together they form potent

Citation Yadav S, Eleftherianos I. 2019. Participation of the serine protease Jonah66Ci in the *Drosophila* antinematode immune response. Infect Immun 87:e00094-19. https://doi.org/10.1128/IAI.00094-19.

Editor De'Broski R. Herbert, University of Pennsylvania

Address correspondence to Ioannis Eleftherianos, ioannise@gwu.edu.

Received 31 January 2019
Returned for modification 24 February 2019
Accepted 29 May 2019
Accepted manuscript posted online 10 June 2019
Published 21 August 2019

pathogenic complexes that infect insects (7, 12). The nematodes enter the insect cavity through the cuticle or natural openings and subsequently expel their bacteria into the insect open circulatory system (15). The bacteria secrete toxins, virulence factors, and degradative enzymes that target several insect tissues and interfere with the insect immune response, which eventually leads to rapid insect death (15). The bacteria also provide nutrients to the nematodes that promote the completion of their reproductive cycle (16). Once the food source is depleted, the nematodes reacquire the bacteria and exit the insect cadaver in search of new insect hosts (12).

The use of *Drosophila* and *Steinernema* to unravel the insect antinematode immune response has certain advantages. Symbiotic and axenic *Steinernema* nematodes are pathogenic to *Drosophila*, and, interestingly, they are capable of killing larvae at similar rates (17). In addition, *Steinernema* nematode infection activates the expression of antimicrobial peptide (AMP) genes and the melanization pathway, and mutualistic *Xenorhabdus* bacteria suppress the latter response (7). The *imaginal disc growth factor-3* (*Idgf3*) and two clotting factors (gp150 and fondue) have been found to participate specifically in the *Drosophila* antinematode immune response. Knockdown of *Idgf3*, *gp150*, or *fondue* increases the susceptibility of larvae responding to *Heterorhabditis bacteriophora* nematodes, whereas inactivation of *Idgf2* provides a survival advantage to larvae responding to axenic *Steinernema* nematodes (18–20).

The *Jonah* multigene family consists of approximately 20 genes organized in small clusters on different chromosomal sites and exhibits complex expression patterns (21–23). *In situ* hybridization identified the expression of *Jonah25Bi*, *Jonah65Ai*, and *Jonah99C*α in the *Drosophila* midgut (24). These *Jonah* genes are expressed during the larval and adult stages of *Drosophila* but not during the pupal stage (21). Low-level *Jonah* expression is also detected in the presumptive midgut from 18-h embryos (23). Because *Jonah* genes are exclusively expressed in the *Drosophila* gut, Jonah proteases are implicated in the breakdown of dietary proteins due to their homology to mammalian serine proteases, trypsin and chymotrypsin (25). More recently, transcriptomic studies have identified the induction of several *Jonah* genes in *Drosophila* responding to viral or nematode infections (8, 26, 27).

In this study, we have investigated the transcriptional regulation of *Jonah66Ci* in *Drosophila* larvae infected with symbiotic or axenic *Steinernema* nematodes. *Jonah66Ci* was selected from a previous transcriptomic study based on its high transcriptional induction in *Drosophila* larvae during *Steinernema* nematode infection (8). In uninfected and nematode-infected larvae, *Jonah66Ci* is solely expressed in the gut (22). To this end, we monitored the survival response, induction of immune signaling pathway effector genes, mitotic rates and numbers of gut cells, levels of nitric oxide (NO) and reactive oxygen species (ROS), and feeding rates in background control and *Jonah66Ci* knockdown larvae. We discuss how inactivation of *Jonah66Ci* in *Drosophila* alters different aspects of the immune response to *Steinernema* and how *Jonah66Ci* is involved in regulating gut physiology against entomopathogenic nematode infection. Identification and functional characterization of genes that are involved in the interaction of *Drosophila* with parasitic nematodes set the stage for uncovering conserved mechanisms in other insects of agricultural or medical importance.

RESULTS

***Steinernema* nematode infection upregulates *Jonah66Ci* in *Drosophila*.** To investigate the transcriptional induction of *Jonah66Ci* in *Drosophila* during nematode infection, we exposed larvae to 100 symbiotic or axenic *Steinernema* nematodes and estimated the relative transcript levels of *Jonah66Ci* at 6 and 24 h postinfection. We compared the transcript levels (as reads per kilobase per million [RPKM]) of *Jonah66Ci* from a recent transcriptomic study (8) and those from quantitative reverse transcription-PCR (qRT-PCR) analysis (ΔC_T method, where C_T is threshold cycle) (Fig. 1A; see also Table S2 in the supplemental material). We have found comparable transcript levels of *Jonah66Ci* by transcriptome sequencing (RNA-seq) and qRT-PCR analyses.

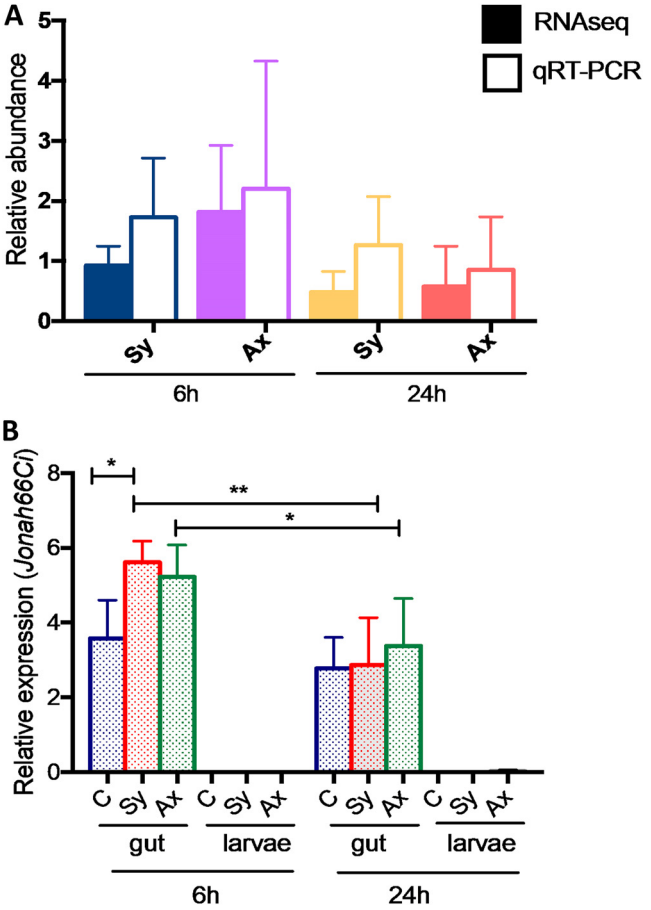

FIG 1 Relative gene transcript levels of *Jonah66Ci* in *Drosophila* larvae upon infection with *Steinernema* nematodes. (A) Relative transcript levels of *Jonah66Ci* using RNA-seq and qRT-PCR analysis were estimated in *Drosophila melanogaster* late-second- or early-third-instar larvae (Oregon line) at 6 and 24 h postinfection with 10 symbiotic (Sy) or axenic (Ax) infective *Steinernema carpocapsae* juveniles. (B) Relative transcript levels for *Jonah66Ci* were estimated in the gut only and in the rest of the larvae in *Drosophila* infected with symbiotic (Sy) or axenic (Ax) *Steinernema* nematodes. Application of water served as a negative-control (C) treatment. Relative gene transcript levels for *Jonah66Ci* were measured as a ratio to the level of the uninfected control samples. Values represent the means from three separate experiments, and error bars represent standard deviations. Data analysis was performed using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test on GraphPad Prism, version 7, software. *, $P < 0.05$; **, $P < 0.01$; nonsignificant differences are not shown.

A previous study identified members of the *Jonah* gene family, *Jonah25Bi*, *Jonah65Ai*, and *Jonah99Cα*, that were expressed in the *Drosophila* gut (21). To determine whether *Jonah66Ci* is also expressed in the gut of *Drosophila* larvae during nematode infection, we estimated the transcript levels of *Jonah66Ci* in *Drosophila* larvae with a gut and without a gut (gutless larvae) at 6 and 24 h postinfection with symbiotic or axenic *Steinernema* nematodes. We detected no mRNA levels of *Jonah66Ci* in the body of gutless nematode-infected or uninfected control larvae (Fig. 1B; Table S2). At 6 h, *Jonah66Ci* transcript levels were significantly higher in the gut of larvae infected with symbiotic nematodes than in uninfected controls ($P = 0.0190$) (Fig. 1B). At 24 h, there were no differences in *Jonah66Ci* transcript levels in the gut of nematode-infected and uninfected larvae. We also found that *Jonah66Ci* transcript levels were significantly reduced from 6 h to 24 h in the gut of larvae infected with symbiotic ($P = 0.0073$) or axenic ($P = 0.0452$) nematodes (Fig. 1B; Table S2). These results indicate that *Jonah66Ci* is expressed at detectable levels in the gut of uninfected *Drosophila* larvae and that challenge with *Steinernema* nematodes leads to upregulation during the early stages of infection.

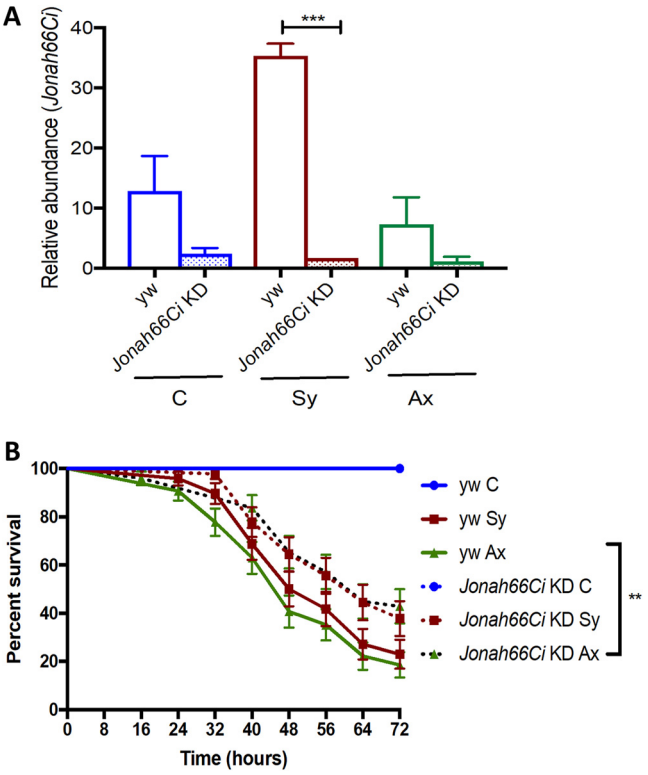

FIG 2 *Drosophila Jonah66Ci* knockdown validation and survival response of *Drosophila Jonah66Ci* knockdown larvae upon infection with *Steinernema* nematodes. (A) Representative figure depicting the relative expression of *Jonah66Ci* in control and *Jonah66Ci* knockdown (KD) larvae following infection with symbiotic (Sy) or axenic (Ax) *Steinernema carpocapsae* nematodes. Water-treated larvae served as negative controls (C). Larval progeny were obtained by crosses involving either female virgin flies from the *yw* background line or from the *Jonah66Ci* RNAi knockdown (KD) line with males from the *Esg*-Gal4 line. Relative gene transcript levels for *Jonah66Ci* were estimated as a ratio to the level of the uninfected control samples. Values represent the means from three independent experiments, and error bars represent standard deviations. Data analysis was performed using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test on GraphPad Prism, version 7, software (***, $P < 0.001$). (B) Survival rates of *Drosophila melanogaster* late-second- or early-third-instar *yw* control and *Jonah66Ci* knockdown larvae following infection with 10 symbiotic (Sy) or axenic (Ax) infective *Steinernema carpocapsae* juveniles. Larval progeny were generated by crossing either female virgin flies from the *yw* background line or *Jonah66Ci* RNAi line with males from the *Esg*-Gal4 line. Application of water served as a control (C) treatment. Survival results were monitored every 8 h and up to 72 h postinfection. Values are shown as percent survival of infected larvae, and data analysis was performed using a log rank (Mantel-Cox) test (GraphPad Prism, version 7 software). The means from three independent experiments are shown, and bars represent standard errors. **, $P < 0.01$; nonsignificant differences are not shown.

***Drosophila Jonah66Ci* knockdown larvae display enhanced survival in response to axenic *Steinernema* nematode infection.** To investigate whether inactivation of *Jonah66Ci* affects the survival ability of *Drosophila* in the context of nematode infection, we challenged *Jonah66Ci* knockdown and *yw* control larvae with symbiotic or axenic *Steinernema* nematodes and assessed larval survival every 8 h for 3 days (Fig. 2B; Table S3). We found that upon infection with axenic nematodes, *yw* control larvae succumbed faster to infection than *Jonah66Ci* knockdown larvae ($P = 0.0028$) (Fig. 2B; Table S3). There were no differences in survival rates between *Jonah66Ci* knockdown and *yw* control larvae infected with symbiotic nematodes ($P = 0.0801$) (Fig. 2B; Table S3). These results indicate that loss of *Jonah66Ci* promotes the survival ability of *Drosophila* larvae in response to axenic *Steinernema* nematode infection.

Imd pathway activation decreases in *Drosophila Jonah66Ci* knockdown larvae responding to axenic *Steinernema* nematodes. To determine whether inactivation of *Jonah66Ci* in *Drosophila* has an effect on signaling pathway activation in response to nematode infection, we infected *Jonah66Ci* knockdown and background control larvae with symbiotic or axenic *Steinernema* nematodes and estimated transcript levels of

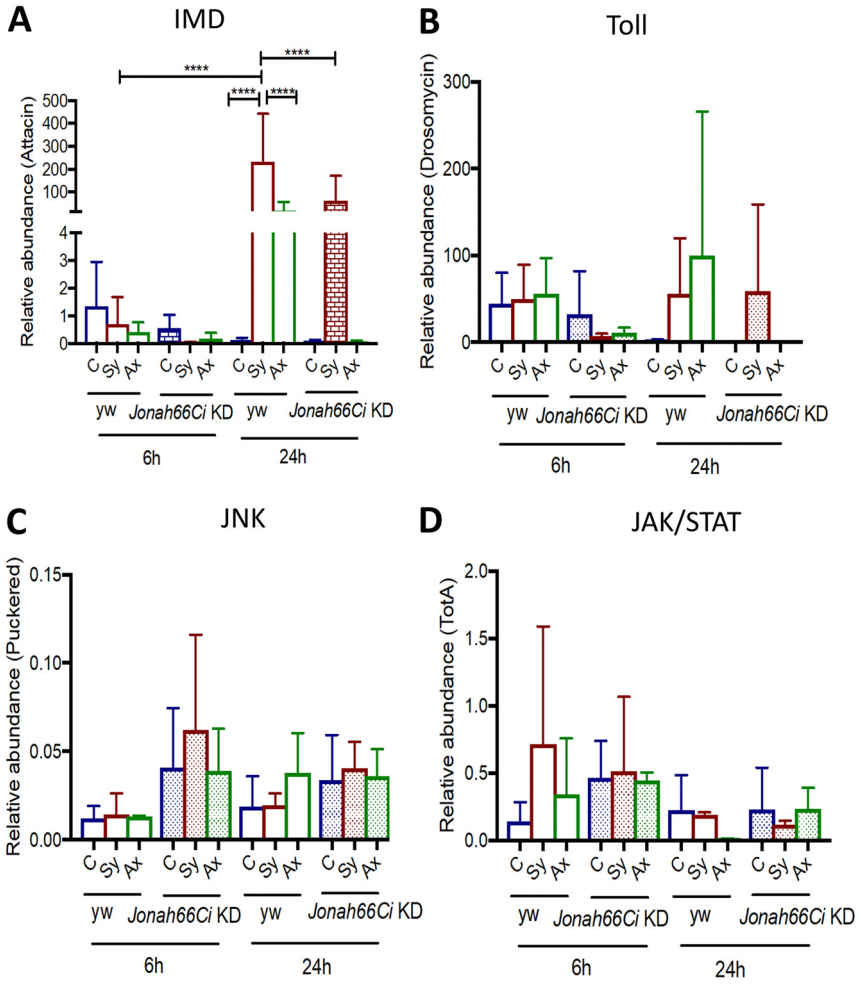

FIG 3 Transcript levels of immune pathway readout genes in *Drosophila Jonah66Ci* knockdown larvae infected with *Steinernema* nematodes. (A to D) Shown are transcript levels of *Attacin* (IMD pathway), *Drosomycin* (Toll pathway), *Puckered* (JNK pathway), and *TotA* (JAK/STAT pathway), as indicated, in *Drosophila melanogaster yw* control and *Jonah66Ci* knockdown larvae 6 and 24 h after being infected with 10 symbiotic (Sy) or axenic (Ax) infective *Steinernema carpocapsae* juveniles or treated with water (control, C). *Drosophila yw* background control and *Jonah66Ci* knockdown (KD) virgin female flies were crossed with *Esg*-Gal4 males, and the resulting larval progeny were used for experiments. Gene transcript values were calculated relative to value for the housekeeping gene, *RpL32*, and expressed as a ratio to the level of the uninfected controls. Samples were run as technical duplicates, and three biological replicates were performed. Bars represent standard deviations. Data analysis was performed using one-way analysis of variance (ANOVA) with a Tukey's *post hoc* test on GraphPad Prism, version 7, software. ****, $P < 0.0001$; nonsignificant differences are not shown.

Attacin (Imd pathway), *Drosomycin* (Toll pathway), *Puckered* (JNK pathway), and *TotA* (Turandot-A, Jak/Stat pathway) at two time points postinfection (Fig. 3; Table S4) (28–31).

At 6 h, we found low transcript levels of *Attacin* in both *yw* controls and *Jonah66Ci* knockdown larvae infected with symbiotic or axenic nematodes. At 24 h, infection with symbiotic nematodes significantly upregulated *Attacin* in *yw* control larvae compared to levels in uninfected individuals ($P < 0.0001$) (Fig. 3A and Table S4). However, we found no differences in *Attacin* mRNA levels in *yw* control larvae infected with axenic nematodes compared to levels in uninfected controls. *Attacin* transcript levels were lower in *yw* control larvae infected with axenic nematodes than in those infected with symbiotic nematodes ($P < 0.0001$) (Fig. 3A and Table S4). We also found that in *yw* control larvae, *Attacin* transcript levels increased significantly from 6 to 24 h after symbiotic nematode infection ($P < 0.0001$) (Fig. 3A and Table S4). At 24 h, *Attacin*

transcript levels in uninfected *Jonah66Ci* knockdown larvae and in those infected with axenic nematodes were hardly detectable. Interestingly, upon symbiotic nematode infections, *Attacin* transcript levels were significantly higher in *yw* control larvae than in *Jonah66Ci* knockdown larvae ($P < 0.0001$) (Fig. 3A and Table S4).

There were no significant differences in transcript levels of *Drosomycin*, *Puckered*, or *Tot-A* between *yw* controls and *Jonah66Ci* knockdown larvae upon infection with symbiotic or axenic nematodes at any of the time points (Fig. 3B to D, respectively; Table S4). These results indicate that the absence of *Jonah66Ci* in *Drosophila* larvae reduces the induction of Imd signaling in response to axenic *Steinernema* nematodes.

Toll and Imd pathways are differentially activated in *Drosophila Jonah66Ci* knockdown larvae responding to symbiotic *Steinernema* nematodes. Toll and Imd pathways regulate antimicrobial peptide production in the anterior midgut of *Drosophila* (32). Restricted expression of *Jonah66Ci* in the *Drosophila* gut (Fig. 1B) prompted us to investigate whether its inactivation would affect Toll or Imd signaling in the context of nematode infection. For this, we infected *Jonah66Ci* knockdown and their background control larvae with symbiotic or axenic *Steinernema* nematodes, and 24 h later we estimated transcript levels of antimicrobial peptide-encoding genes in the gut and the rest of the larva (Fig. 4; Table S5).

Low transcript levels of *Drosomycin* were detected in *Jonah66Ci* knockdown larvae, with or without nematode infection. We found significantly elevated levels of *Drosomycin* in the gut of *yw* control larvae infected with symbiotic nematodes compared to levels in those infected with axenic nematodes ($P = 0.005$) and in uninfected controls ($P = 0.0006$) (Fig. 4A and Table S5). In *yw* control larvae infected with symbiotic *Steinernema* nematodes, *Drosomycin* transcript levels were significantly higher in the gut than in gutless larvae ($P = 0.0391$) (Fig. 4A and Table S5). Most importantly, *Drosomycin* transcript levels were higher in the gut of *yw* control larvae than in the gut of *Jonah66Ci* knockdown larvae infected with symbiotic nematodes ($P = 0.0004$) (Fig. 4A and Table S5).

In contrast, we found significantly higher levels of *Defensin* in gutless *Jonah66Ci* knockdown larvae responding to symbiotic nematodes than in uninfected larvae ($P = 0.0152$) (Fig. 4B and Table S5). *Defensin* in gutless *Jonah66Ci* larvae was also significantly higher than in the gut of the knockdown larvae ($P = 0.171$) and the control gutless larvae ($P = 0.0188$) in response to symbiotic nematode infection (Fig. 4B; Table S5).

Infection with symbiotic nematodes consistently increased *Diptericin* in the gut and in gutless *yw* control and *Jonah66Ci* knockdown larvae (Fig. 4C; Table S5). *Diptericin* was significantly higher in control gutless larvae responding to symbiotic nematode infection than in uninfected gutless larvae ($P = 0.0203$), in those infected with axenic nematodes ($P = 0.0198$), or in gutless *Jonah66Ci* knockdown larvae infected with symbiotic nematodes ($P = 0.0441$) (Fig. 4C and Table S5).

Interestingly, *Cecropin* was significantly upregulated in the gut of *Jonah66Ci* knockdown larvae infected with symbiotic nematodes compared to levels in those infected with axenic nematodes ($P = 0.0001$) and in uninfected control larval gut ($P = 0.0002$) (Fig. 4D and Table S5). This increase was also statistically significant compared to *Cecropin* levels in gutless *Jonah66Ci* knockdown larvae infected with symbiotic nematodes ($P = 0.0005$) as well as to levels in the gut of *yw* control larvae infected with symbiotic nematodes ($P = 0.0002$) (Fig. 4D and Table S5). *Cecropin* was upregulated in control gutless larvae upon infection with symbiotic nematodes, but this increase was not statistically significant (Fig. 4D; Table S5). These results demonstrate that the absence of *Jonah66Ci* in *Drosophila* larvae leads to differential expression of the Toll and Imd pathway-regulated antimicrobial peptide genes in the gut and the rest of the larval body in response to infection with *Steinernema* nematodes.

Mitosis is reduced in *Drosophila Jonah66Ci* knockdown larvae in response to symbiotic *Steinernema* nematodes. Because *Jonah66Ci* is entirely expressed in the gut of *Drosophila* larvae, we explored whether the absence of *Jonah66Ci* influences the activation of the gut-specific Wnt/Wg signaling pathway, which regulates gut tissue homeostasis during development (33, 34) (Fig. 5). For this, we infected *yw* background control and *Jonah66Ci* knockdown larvae with symbiotic or axenic *Steinernema* nem-

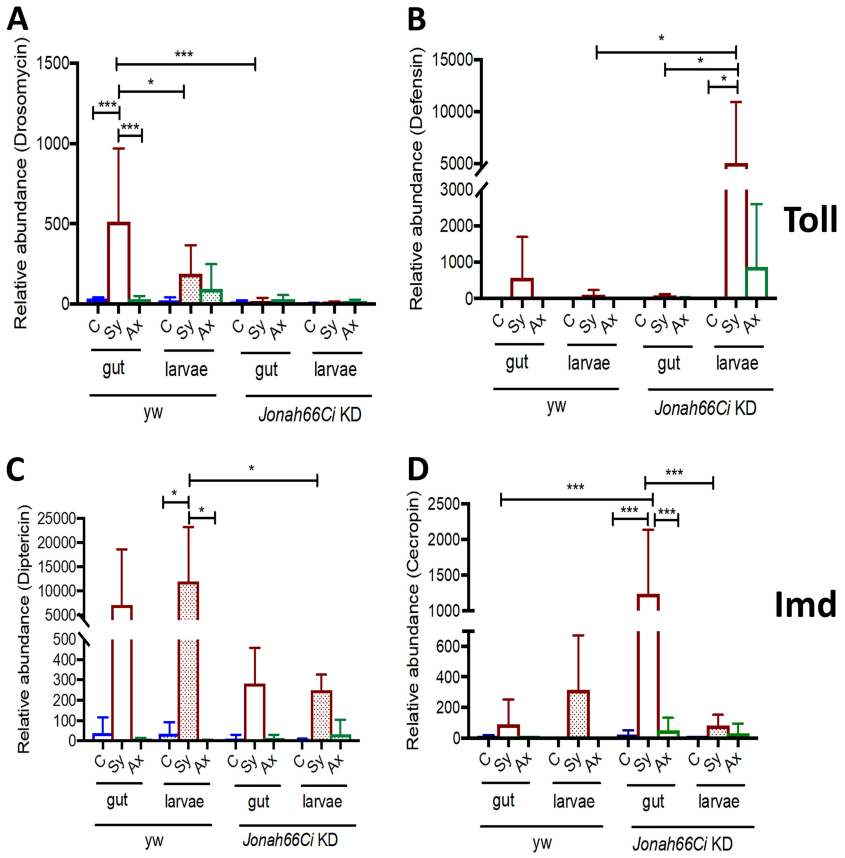

FIG 4 Transcript levels of Toll and Imd pathway readout genes in the gut of *Drosophila Jonah66Ci* knockdown larvae infected with *Steinernema* nematodes. Shown are transcript levels of *Drosomycin* (A), *Defensin* (Toll pathway) (B), *Diptericin* (C), and *Cecropin* (IMD pathway) (D) in *Drosophila yw* control and *Jonah66Ci* knockdown larvae 24 h after being infected with 10 symbiotic (Sy) or axenic (Ax) infective *Steinernema carpocapsae* juveniles or treated with water (control, C). *Drosophila yw* background control and *Jonah66Ci* knockdown (KD) virgin female flies were crossed with *Esg*-Gal4 males, and the resulting larval progeny were used for experiments. Gene transcript levels are shown in gut tissue only and the rest of the larvae. Transcript level values are calculated relative to the level of the housekeeping gene, *RpL32*, and are expressed as a ratio to level of uninfected control samples. Three independent experiments were performed, and bars represent standard deviations. Data analysis was performed using one-way analysis of variance (ANOVA) with a Tukey's *post hoc* test on GraphPad Prism, version 7, software. *, $P < 0.05$; ***, $P < 0.001$; nonsignificant differences are not shown.

atodes and estimated transcript levels of *wingless*, encoding a ligand of the Wnt/Wg signaling pathway, in the gut 24 h postinfection. We found that *wingless* was significantly upregulated in the gut of uninfected *Jonah66Ci* knockdown larvae compared to the level in the control line (Fig. 5A; Table S6). We also found that upon symbiotic nematode infection, *wingless* was upregulated in the gut of *Jonah66Ci* knockdown larvae compared to the level in *yw* control larvae (Fig. 5A; Table S6). However, we found no significant differences in *wingless* transcript levels between *Jonah66Ci* larvae infected with symbiotic and axenic nematodes or between nematode-infected and control larvae. Additionally, infection with symbiotic or axenic nematodes had no effect on *wingless* transcript levels in the gut of *yw* control larvae. Thus, these results suggest that inactivation of *Jonah66Ci* upregulates Wnt/Wg signaling in the gut of *Drosophila* larvae in the presence or absence of nematode infection.

Wnt/Wg signaling promotes tissue regeneration in the *Drosophila* gut after injury (35). To investigate whether inactivation of *Jonah66Ci* in the gut affects tissue regeneration in response to nematode infection, we infected *yw* background control and *Jonah66Ci* knockdown larvae with symbiotic or axenic *Steinernema* nematodes and measured the number of mitotic cells (phospho-histone H3 [PH3] labeled) in the gut of

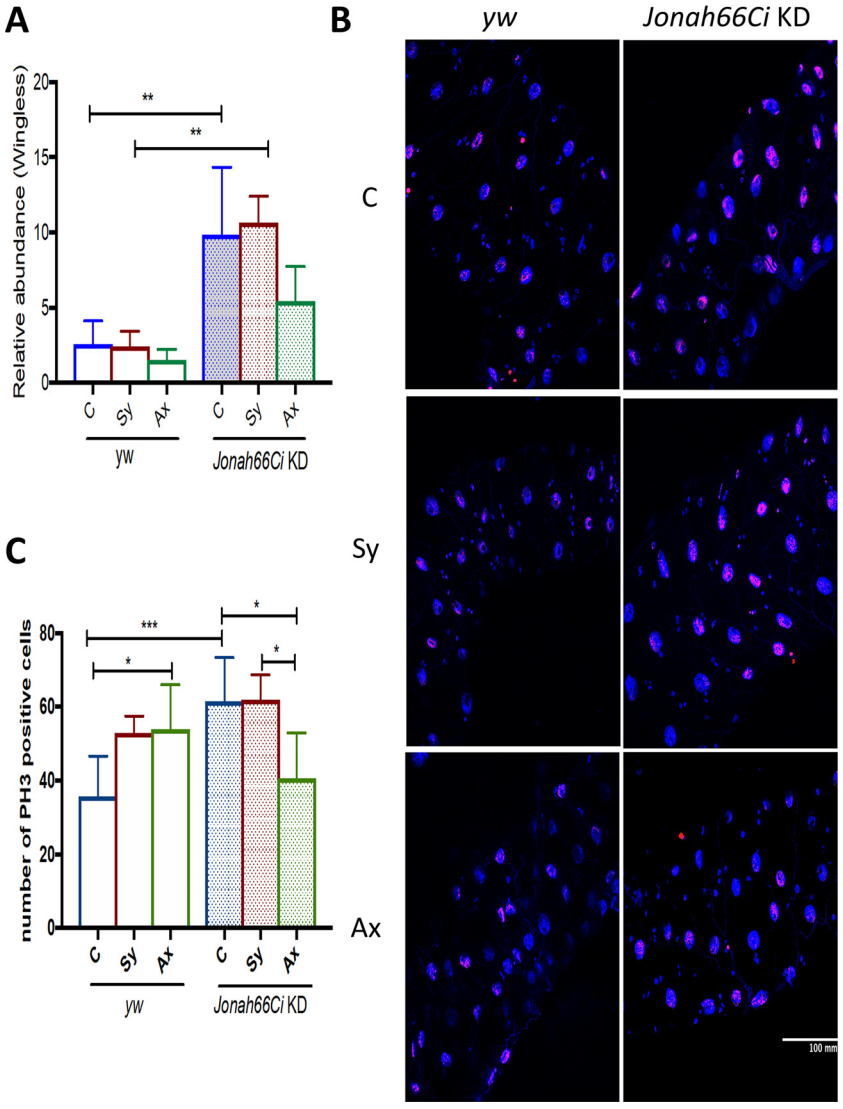

FIG 5 Mitosis in the intestinal cells of *Drosophila Jonah66Ci* knockdown larvae infected with *Steinernema* nematodes. (A) Relative *wingless* transcript levels. (B) Representative images of gut cells labeled with phospho-histone 3 (PH3; red) and DAPI (blue) at ×40 magnification. (C) Number of mitotic cells in the gut of *Drosophila melanogaster yw* control and *Jonah66Ci* knockdown larvae at 24 h postinfection with 10 symbiotic (Sy) or axenic (Ax) infective *Steinernema carpocapsae* juveniles. Water-treated larvae served as controls (C). *Drosophila yw* background control and *Jonah66Ci* knockdown (KD) virgin female flies were crossed with *Esg*-Gal4 males, and the resulting larval progeny were used for experiments. Transcript levels were estimated relative to the levels of the housekeeping gene, *RpL32*, and as a ratio to the level of uninfected control larvae. All experiments were repeated three times, and data analysis was performed using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test on GraphPad Prism, version 7. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; nonsignificant differences are not shown.

infected and uninfected individuals (Fig. 5B). In uninfected guts, the numbers of PH3-labeled cells significantly increased in *Jonah66Ci* knockdown larvae compared to levels in the *yw* controls ($P = 0.008$) (Fig. 5B and C and Table S6). Interestingly, infection of *yw* controls with axenic nematodes significantly increased the numbers of PH3-labeled cells compared to the levels in uninfected control larvae ($P = 0.0278$) (Fig. 5B and C and Table S6). Also, inactivation of *Jonah66Ci* significantly reduced the numbers of PH3-labeled cells in the gut of larvae infected with axenic nematodes compared to levels in uninfected individuals ($P = 0.0131$) (Fig. 5B and C and Table S6). Additionally, the numbers of PH3-labeled cells were significantly lower in the gut of *Jonah66Ci* knockdown larvae infected with axenic nematodes than in those infected with symbi-

otic nematodes ($P = 0.0148$) (Fig. 5B and C and Table S6). Thus, under normal conditions, the absence of *Jonah66Ci* in *Drosophila* larvae increases the numbers of gut cells undergoing mitosis, and this effect is reduced in response to infection with axenic *Steinernema* nematodes.

Enteroendocrine cell numbers are reduced in *Drosophila Jonah66Ci* knockdown larvae in response to *Steinernema* nematode infection. Because inactivation of *Jonah66Ci* increases the numbers of mitotic cells in the gut of uninfected *Drosophila* larvae, we investigated whether *Jonah66Ci* inactivation also affects the specific cell types of the larval gut in the presence or absence of nematode infection. For this, we infected *yw*-background control and *Jonah66Ci* knockdown larvae with symbiotic or axenic *Steinernema* nematodes and estimated the numbers of enteroendocrine (EE) cells (Prospero labeled) in the gut of infected and uninfected individuals (Fig. 6; Table S7). In uninfected guts, the numbers of EE cells were significantly higher in *Jonah66Ci* knockdown larvae than in *yw* control larvae ($P = 0.0048$) (Fig. 6B and Table S7). Interestingly, infection of *yw* control larvae with symbiotic nematodes increased significantly the numbers of EE cells compared to levels in uninfected control larvae ($P = 0.0286$) (Fig. 6B and Table S7) and in *yw* control larvae infected with axenic nematodes ($P = 0.0179$) (Fig. 6B and Table S7). Conversely, inactivation of *Jonah66Ci* reduced significantly the numbers of EE cells in the gut of larvae infected with symbiotic or axenic nematodes compared to levels in uninfected controls ($P = 0.0182$ or $P = 0.0131$, respectively) (Fig. 6B and Table S7). Thus, under normal conditions, inactivation of *Jonah66Ci* in *Drosophila* larvae increases the numbers EE cells, which are conversely reduced in response to *Steinernema* nematode infection.

Intestinal stem cell numbers are unaffected in *Drosophila Jonah66Ci* knockdown larvae in response to *Steinernema* nematode infection. Because inactivation of *Jonah66Ci* increases the numbers of EE cells as well as the numbers of cells undergoing mitosis in uninfected larvae, we investigated whether inactivation of *Jonah66Ci* in the gut affects the stem cell population in the presence or absence of nematode infection. For this, we infected *yw* background control and *Jonah66Ci* knockdown larvae with symbiotic or axenic *Steinernema* nematodes and assessed the number of intestinal stem cells (ISCs; escargot labeled) in the gut of infected and uninfected individuals (Fig. 7; Table S8). Interestingly, we found no changes in the ISC populations in the gut of *yw* control larvae and *Jonah66Ci* knockdown larvae, with or without nematode infection. These results suggest that inactivation of *Jonah66Ci* in *Drosophila* larvae infected with *Steinernema* nematodes has no effect on the numbers of ISCs.

NO, but not ROS or feeding, is reduced in *Drosophila Jonah66Ci* knockdown larvae in response to symbiotic *Steinernema* nematodes. To determine whether certain physiological processes in *Drosophila* are affected by the absence of *Jonah66Ci* in the context of nematode infection, we measured nitric oxide (NO) and reactive oxygen species (ROS) levels as well as feeding rates at 24 h postinfection of larvae with symbiotic or axenic *Steinernema* nematodes (Fig. 8; Table S9). We found that nitric oxide levels increased in *yw* control larvae infected with symbiotic nematodes compared to levels in those infected with axenic nematodes ($P = 0.0320$) and to those in *Jonah66Ci* knockdown larvae infected with symbiotic worms ($P = 0.0123$) (Fig. 8A and Table S9). There were no changes in nitric oxide in *Jonah66Ci* knockdown larvae in the presence or absence of nematode infection. We also measured ROS levels by estimating the relative aconitase activity in *yw* control and *Jonah66Ci* knockdown larvae at 24 h post-nematode infection. We found no changes in aconitase activity levels between *yw* control and *Jonah66Ci* knockdown larvae upon nematode infection or under normal conditions ($P > 0.05$) (Fig. 8B and Table S9). We also measured the feeding rates in *yw* control and *Jonah66Ci* knockdown larvae at 24 h post-nematode infection. We found that feeding rates increased in background control larvae upon infection with axenic *Steinernema* and that this increase was significantly higher than that in *yw* control larvae upon infection with symbiotic nematodes ($P = 0.0204$) and that in *Jonah66Ci* knockdown larvae upon infection with axenic worms ($P = 0.0249$) (Fig. 8C and D and

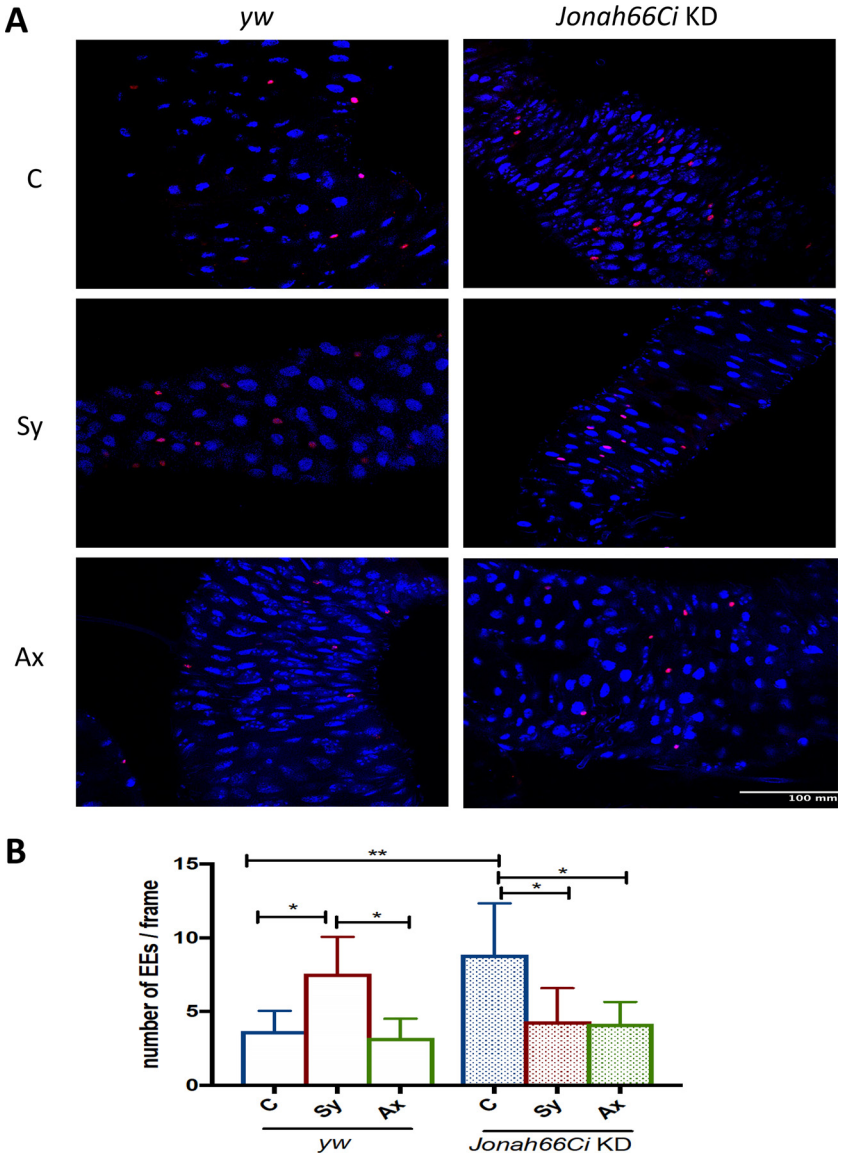

FIG 6 Enteroendocrine cell numbers in *Drosophila Jonah66Ci* knockdown larvae infected with *Steinernema* nematodes. (A) Representative images of gut cells labeled with Prospero (red) and DAPI (blue) at ×40 magnification. (B) Number of enteroendocrine cells in the gut of *Drosophila melanogaster yw* control and *Jonah66Ci* knockdown (KD) larvae at 24 h postinfection with 10 symbiotic (Sy) or axenic (Ax) infective *Steinernema carpocapsae* juveniles. Water-treated larvae served as controls (C). *Drosophila yw* background control and *Jonah66Ci* knockdown virgin female flies were crossed with *Esg*-Gal4 males, and the resulting larval progeny were used for experiments. All experiments were repeated three times, and data analysis was performed using one-way analysis of variance (27) with Tukey's *post hoc* test on GraphPad Prism, version 7. *, $P < 0.05$; **, $P < 0.01$; nonsignificant differences are not shown.

Table S9). These results indicate that inactivation of *Jonah66Ci* decreases NO levels in *Drosophila* larvae upon symbiotic *Steinernema* nematode infection.

DISCUSSION

In this study, we investigated the immune and pathophysiological effects of *Jonah66Ci* in *Drosophila* larvae in the context of nematode infection. First, we showed that *Jonah66Ci* is expressed in the gut of *Drosophila* larvae in the presence or absence of *Steinernema* nematode infection. Then, we monitored the survival ability of wild-type and *Jonah66Ci* knockdown larvae in response to *Steinernema* nematode infection. We also evaluated the differential induction of the Toll and Imd pathway effector genes, quantified the mitotic cells in the gut, estimated the numbers of EE cells and ISCs, and

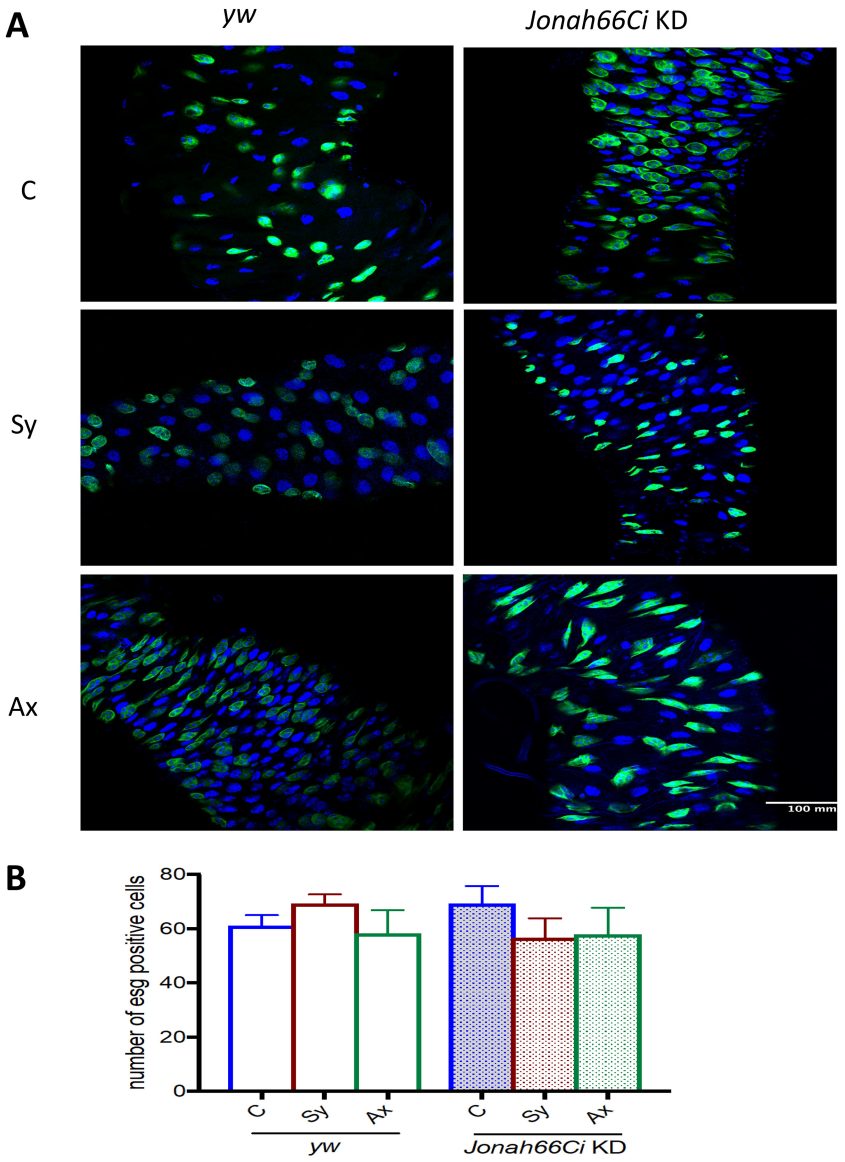

FIG 7 Intestinal stem cell numbers in *Drosophila Jonah66Ci* knockdown larvae infected with *Steinernema* nematodes. (A) Representative images of larval guts expressing the *esg→gfp* driver (green) and labeled with DAPI (blue) at ×40 magnification. (B) Number of intestinal stem cells in the gut of *Drosophila melanogaster yw* control and *Jonah66Ci* knockdown larvae at 24 h postinfection with 10 symbiotic (Sy) or axenic (Ax) infective *Steinernema carpocapsae* juveniles. Water-treated larvae served as controls (C). *Drosophila yw* background control and *Jonah66Ci* knockdown (KD) virgin female flies were crossed with *Esg*-Gal4 males, and the resulting larval progeny were used for experiments. All experiments were repeated three times, and data analysis was performed using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test on GraphPad Prism, version 7. Differences were nonsignificant.

measured the NO and ROS activity levels as well as feeding rates of wild-type and *Jonah66Ci* knockdown larvae during symbiotic or axenic nematode infection or under normal conditions. We report that the serine protease-encoding gene *Jonah66Ci* plays an essential role in maintaining homeostasis in the gut of *Drosophila* larvae upon infection with a potent nematode parasite.

Because we detected expression of *Jonah66Ci* only in the gut of uninfected and nematode-infected *Drosophila* larvae, we hypothesized that *Jonah66Ci* controls physiological processes and signaling pathways specific to this tissue. We found that inactivation of *Jonah66Ci* in the gut of *Drosophila* larvae responding to symbiotic or axenic *Steinernema* nematode infection resulted in differential expression of the Toll

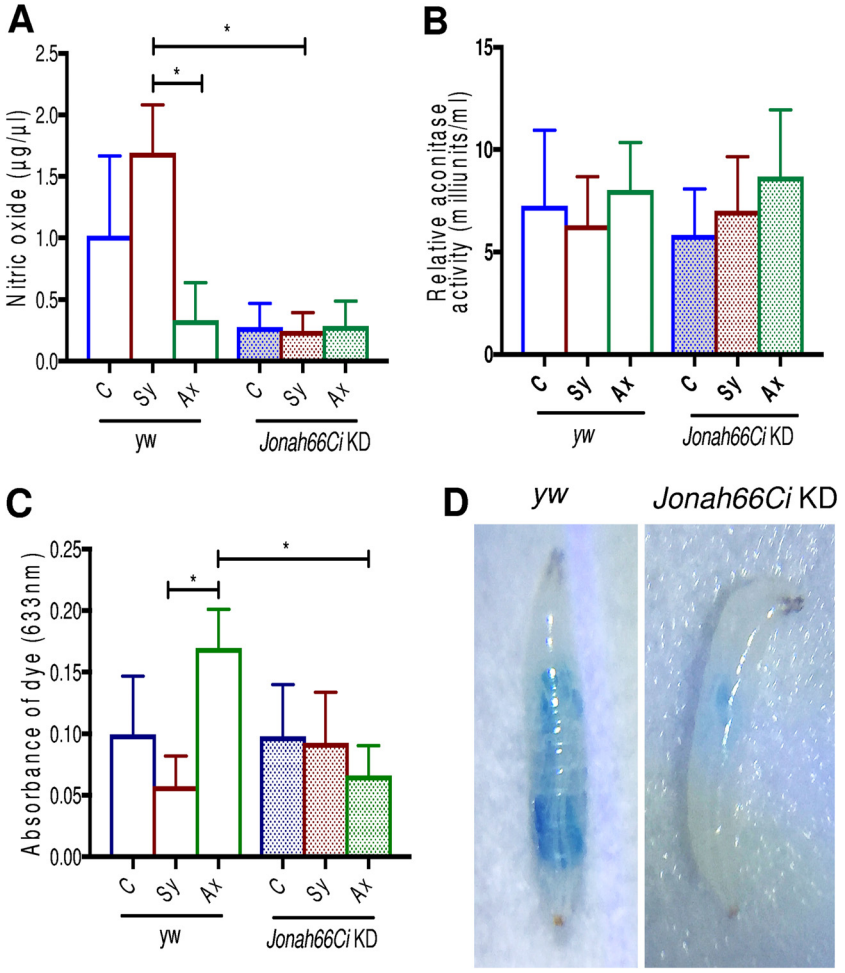

FIG 8 Nitric oxide and aconitase activity levels and feeding rates in *Drosophila Jonah66Ci* knockdown larvae infected with *Steinernema* nematodes. (A and B) Relative nitric oxide (NO) and aconitase activity levels in the gut. (C) Spectrophotometric analysis of food intake in *Drosophila melanogaster yw* control and *Jonah66Ci* knockdown larvae at 24 h postinfection with symbiotic (Sy) or axenic (Ax) infective *Steinernema carpocapsae* juveniles. Water-treated larvae served as controls (C). (D) Feeding rate of *yw* control and *Jonah66Ci* knockdown larvae at 24 h postinfection with axenic *S. carpocapsae* nematodes. *Drosophila yw* background control and *Jonah66Ci* knockdown (KD) virgin female flies were crossed with *Esg*-Gal4 males, and the resulting larval progeny were used for experiments. NO and aconitase activity levels were measured relative to total protein. Experiments were repeated three times and analyzed using one-way analysis of variance (ANOVA) with a Tukey's *post hoc* test on GraphPad Prism, version 7. *, $P < 0.05$; nonsignificant differences are not shown.

pathway readout genes *Drosomycin* and *Defensin* and of the Imd pathway genes *Diptericin* and *Cecropin*. A previous study reported an increase in expression of *Drosomycin* and *Diptericin* in *Drosophila* flies injected with *Xenorhabdus,* the mutualistic bacterium of *Steinernema* nematodes (36). Our data are in agreement with this study since we found upregulation of these two AMP-encoding genes in the gut of *Drosophila yw* control larvae responding to *Steinernema* nematodes carrying mutualistic *Xenorhabdus* bacteria compared to levels in larvae responding to axenic nematodes. In contrast, we found reduced expression of *Drosomycin* and *Diptericin* in the gut of *Jonah66Ci* knockdown larvae infected with the same type of nematode, implying that *Jonah66Ci* regulation of the Toll and Imd signaling activities in the gut of *Drosophila* infected with *Steinernema* nematodes is closely associated with the presence of *Xenorhabdus* bacteria. This finding is in agreement with a previous report indicating that expression of Toll and Imd effector genes in *Drosophila* is higher in the case of symbiotic nematode infections than in axenic nematode infections, which is probably due to the effect of *Xenorhabdus* bacteria on the insect host (7, 37). We also found that

Defensin is upregulated in the *Jonah66Ci* gutless knockdown larvae infected with axenic *Steinernema* and is found at even higher levels in infection with symbiotic nematodes. However, both types of nematodes fail to upregulate this AMP in guts of wild-type larvae or gutless individuals (7). This suggests that *Jonah66Ci* interacts closely with Toll signaling in larvae responding to *Steinernema* nematode infections. Interestingly, *Defensin* is upregulated in *thioester-containing protein-4* mutant flies responding to *Photorhabdus luminescens* or *Photorhabdus asymbiotica* bacterial infection, and this correlates with resistance of the mutant flies to infection (38). Our findings are in agreement with the results in this previous study as we found upregulation of *Defensin* in *Jonah66Ci* knockdown larvae following axenic *Steinernema* nematode infection, which was accompanied by higher survival of the knockdown larvae. The differential induction of Toll and Imd pathway effector genes suggests that inactivation of *Jonah66Ci* regulates immune signaling not only in the gut of *Drosophila* larvae but also in other immune tissues, probably the fat body or hemolymph, which might alter the survival response against parasitic nematode infection.

The *Drosophila* midgut is lined with approximately 2,000 ISCs. These multipotent ISCs give rise to two types of differentiated daughter cells, the secretory enteroendocrine cells and the absorptive enterocytes. Collectively these cells form the monolayer that line the *Drosophila* midgut (39–41). In the case of gut epithelial damage or stress such as bacterial infection, ISCs are able to produce new cells to replace the damaged epithelial cells and regenerate the gut (42). A previous study using *Drosophila* adult flies has indicated that inactivation of *adenomatous polyposis coli* (*Apc*), a tumor suppressor gene found in the intestinal epithelium, results in a significant increase in the numbers of cells undergoing mitosis (39). This finding agrees with our data since we also found that, in the absence of *Steinernema* nematode infection, the numbers of cells undergoing mitosis are significantly increased in the gut of *Jonah66Ci* knockdown larvae (Fig. 5B and C; see also Table S6 in the supplemental material). Thus, this suggests that *Jonah66Ci*, similar to *Apc* in *Drosophila* adults, is essential in maintaining homeostasis in the gut of *Drosophila* larvae responding to *Steinernema* nematodes. The upregulation of *wingless* in uninfected *Jonah66Ci* knockdown larvae also suggests that *Jonah66Ci* expression in the gut interferes with Wnt/Wg pathway activity in regulating cell proliferation (43). Additionally, in the absence of nematode infection, we found that the EE cell numbers were significantly increased in *Jonah66Ci* knockdown larvae compared to levels in background control larvae. This finding agrees with a previous report that loss of catalase function in *Drosophila* adult flies, Cat^{n1} mutants, resulted in higher EE cell numbers (44). Thus, this suggests that the serine protease-encoding gene *Jonah66Ci*, similar to catalase in adult midguts, is responsible for maintaining gut integrity in *Drosophila* larvae.

Contrary to the changes in cell numbers observed above, the numbers of ISCs remained unchanged between control and *Jonah66Ci* knockdown larvae in the presence or absence of nematode infection. This led us to speculate that *Jonah66Ci* likely functions downstream of the ISCs. Further studies will have to be performed to identify the specific role of *Jonah66Ci* in stem cell signaling events. A previous study reported that *Pseudomonas entomophila* secretes hemolysin that targets and lyses the enterocytes in the gut epithelium of *Drosophila* adults and larvae (45, 46). Similarly, *Steinernema* nematodes secrete a serine protease, sc-sp-1, that functions as a virulence factor that disarms the immune system by destroying the gut lumen (47). Hence, we speculate that reduction in the numbers of mitotic and EE cells in the gut of *Jonah66Ci* knockdown larvae responding to *Steinernema* nematodes could be attributed to the virulence factors produced by *Steinernema* nematodes.

A previous study has reported the crucial role of NO in eliminating the eggs of the endoparasitic wasp *Leptopilina heterotoma* in *Drosophila paramelanica* larvae (48). Also, NO is essential for the survival of *Drosophila* flies responding to Gram-negative bacterial infection (49). We found increased NO levels in *yw* control larvae responding to symbiotic, but not axenic, *Steinernema* nematodes. This suggests that *Drosophila* larvae are capable of inducing a NO response against the mutualistic *Xenorhabdus* bacteria.

We also found a reduction in NO levels in the gut of uninfected and nematode-infected *Jonah66Ci* knockdown larvae, suggesting a role for *Jonah66Ci* in regulating the NO antinematode response in *Drosophila* larvae.

In addition to NO, ROS has long been recognized to defend hosts against pathogen infection due to its cytotoxicity (50). *Ecc15* oral infection has been shown to induce ROS stress in *Drosophila* adult flies, and ROS is known to control microbial growth in the host (50, 51). Altogether, ROS has been attributed to playing an important role in initiating immunological communications from gut to fat body in *Drosophila*. However, in the case of nematode infections, we observed no changes in ROS levels in background control or *Jonah66Ci* knockdown larvae. This finding demonstrates that *Jonah66Ci* plays no role in regulating ROS activity in the gut of *Drosophila* larvae in the context of *Steinernema* nematode infection.

We further found reduced feeding rates in *Jonah66Ci* knockdown larvae responding to axenic *Steinernema* nematodes. Infection of adult *Drosophila* with *Drosophila* C virus (DCV) increases the feeding rate of flies (27). Our data agree with this finding since we also found that *yw* control larvae responding to axenic *Steinernema* nematodes ingest significantly larger amounts of food. Because this effect is reduced in *Jonah66Ci* knockdown larvae upon axenic nematode infection, we postulate that *Jonah66Ci* is essential in regulating the food uptake of larvae during infection with parasitic nematodes.

In conclusion, we found that *Jonah66Ci* regulates certain gut-specific responses in *Drosophila* larvae responding to *Steinernema* infection. We showed that the absence of *Jonah66Ci* confers partial protection to larvae against axenic nematodes. We also showed that *Jonah66Ci* differentially induces the effector genes of *Drosophila* Toll and Imd signaling in the gut of larvae responding to symbiotic or axenic *Steinernema* nematodes. Finally, we showed that *Jonah66Ci* regulates gut-specific processes, including immune signaling, the numbers of mitotic and EE cells, and nitric oxide levels in response to nematode attack. Our findings demonstrate a novel function for the *Drosophila* serine protease *Jonah66Ci* in regulating the insect immune response to potent nematode parasites. Similar findings will pave the way toward a better understanding of the tissue-specific molecular players that modulate the insect immune response against parasitic nematodes.

MATERIALS AND METHODS

Fly lines. *Drosophila melanogaster yellow white* (*yw*) and *Jonah66Ci* (v103008, FBst0474871; Vienna *Drosophila* Resource Centre) lines were used. Female flies from the *Jonah66Ci* RNA interference (RNAi) line were crossed with males from the *Escargot* (*Esg*)-Gal4 driver (*w**; P{enG}*esg*^{G66}/CyO, P{GAL4-Kr.C}DC3, P{UAS-GFP.S65T}DC7) (where UAS is upstream activation sequence and GFP is green fluorescent protein) (52). The knockdown of *Jonah66Ci* was validated using the *Esg*-Gal4 line (Fig. 2A). All lines were reared on *Drosophila* medium (Meidi Laboratories) and sprinkled with approximately 10 g of *Saccharomyces cerevisiae* (baker's yeast). Stocks were maintained in a 12/12-h light/dark cycle at 25°C. Late-second- to early-third-instar larvae were used for all experiments.

Nematodes stocks. Infective juveniles of *Steinernema carpocapsae* nematodes were used for all experiments. Symbiotic nematodes carrying *Xenorhabdus nematophila* bacteria were reared in larvae of the wax moth *Galleria mellonella*, as described previously (53). Axenic nematodes lacking *Xenorhabdus* were generated according to a previously established protocol (17). Axenic nematodes were washed in 1% bleach solution to remove bacteria from the nematode surface and rinsed five times with water to remove the bleach residue. Infective juveniles 2 to 5 weeks old were used for all experiments.

Gene transcript analysis with RNA-sequencing. The number of reads per kilobase per million mapped reads (RPKM) for *Jonah66Ci* (locus *CG7118*) were obtained from a recent RNA sequencing study (8). The reads were obtained at 6 and 24 h postinfection of *D. melanogaster* Oregon larvae with 100 symbiotic or axenic infective *S. carpocapsae* juveniles. The RPKM values for nematode-infected larvae are shown relative to the RPKM values of uninfected control larvae at each time point.

Gene transcript analysis with quantitative RT-PCR. Four larvae, each infected with 100 symbiotic or axenic *Steinernema* nematodes, were collected at 6 and 24 h postinfection for analyzing gene transcript levels using qRT-PCR. For estimating *Jonah66Ci* transcript levels in the gut, 10 larvae infected with 10 symbiotic or axenic nematodes were dissected at 6 and 24 h postinfection to separate the gut tissues from the rest of the larvae. In all cases, larvae treated with sterile distilled water served as the uninfected controls. Total RNA extraction was performed using Invitrogen/Ambion TRIzol reagent according to the manufacturer's instructions. RNA extraction, cDNA synthesis, and qRT-PCR protocols were performed as described before (54). All primer sets used for qRT-PCR analyses and their respective annealing temperatures are listed in Table 1. Data were measured from technical duplicates, expressed

TABLE 1 Primers used for quantitative RT-PCR

Gene	Locus	Primer name	Sequence (5′–3′)	T_m (°C)[a]
Jonah66Ci	*CG7118*	Forward	TTCATCACCCACGGATCTGC	57
		Reverse	GCACTCGGAGTTGTGGATGA	
Attacin-A	*CG10146*	Forward	CAATGGCAGACACAATCTGG	60
		Reverse	ATTCCTGGGAAGTTGCTGTG	
Drosomycin	*CG10810*	Forward	TGAGAACCTTTTCCAATATGATG	60
		Reverse	CCAGGACCACCAGCAT	
Puckered	*CG7850*	Forward	GGCCTACAAGCTGGTGAAAG	60
		Reverse	AGTTCAGATTGGGCGAGATG	
Turandot-A	*CG31509*	Forward	AGATCGTGAGGCTGACAAC	60
		Reverse	CCTGGGCGTTTTTGATAA	
Defensin	*CG1385*	Forward	CGCATAGAAGCGAGCCACATG	60
		Reverse	GCAGTAGCCGCCTTTGAACC	
Diptericin	*CG12763*	Forward	ACCGCAGTACCCACTCAATC	60
		Reverse	CCCAAGTGCTGTCCATATCC	
Cecropin-A1	*CG1365*	Forward	TCTTCGTTTTCGTCGCTCTC	60
		Reverse	CTTGTTGAGCGATTCCCAGT	
Wingless	*CG4889*	Forward	GATTATTCCGCAGTCTGGTC	60
		Reverse	CTATTATGCTTGCGTCCCTG	
RpL32	*CG7939*	Forward	GATGACCATCCGCCCAGCA	60
		Reverse	CGGACCGACAGCTGCTTGGC	

[a]T_m, melting temperature.

as the ΔC_T of $2^{CT(RpL32)}/2^{CT(gene)}$ and presented as a ratio of the value for infected larvae to that of the uninfected controls. Results depict mean and standard deviations from three biological replicates representing three independent experiments.

Survival experiments. A 96-well microtiter plate (Corning) was prepared by addition of 100 μl of 1.5% agarose gel (in 1× Tris-acetate-EDTA [TAE] buffer) to each well. A suspension (10 μl) containing 10 symbiotic or axenic *Steinernema* nematodes was added to each well. Application of sterile distilled water (10 μl) to larvae was used as an uninfected control treatment. An individual *Drosophila* larva was then added to each well, as described previously (8). For each experiment, 20 larvae per line per treatment were used, and each survival assay was repeated three times.

Immunohistochemistry. *Drosophila yw* background and *Jonah66Ci* knockdown larvae were collected from five separate vials for each experiment. Gut samples from 10 larvae infected with 10 symbiotic or axenic *Steinernema* nematodes were dissected at 24 h postinfection. Gut samples from larvae treated with sterile distilled water served as uninfected controls. Gut tissues were fixed in 4% formaldehyde (Sigma) in 1× phosphate-buffered saline (PBS) for 30 min and then washed in 1× PBS containing 0.1% Triton X-100. Samples were incubated with primary and secondary antibodies in a solution consisting of 1× PBS, 0.1% Triton X-100, and 0.5% bovine serum albumin (BSA). The following primary antibodies were used: 1:500 rabbit anti-PH3 (Developmental Studies Hybridoma Bank [DSHB]) and mouse anti-Prospero (DSHB). Fluorescently labeled tissues were mounted in ProLong Diamond antifade mountant containing 4′,6′-diamidino-2-phenylindole (DAPI) nuclear stain (Life Technologies). Data were collected from gut tissues from each individual larva. Fluorescent images were obtained using an LSCM-510 Meta confocal microscope (Carl Zeiss) at ×40 magnification. Images were assembled using Adobe Photoshop (2018 release), and numbers of specific cell types (PH3 for mitotic cells, Prospero for EEs, and Gal4-UAS-GFP for ISCs) were estimated. The experiment was repeated two times.

NO and aconitase activity estimation. Gut samples from 10 *Drosophila* larvae infected with 10 symbiotic or axenic *Steinernema* nematodes were dissected 24 h postinfection. Gut samples from larvae treated with sterile distilled water served as uninfected controls. For nitric oxide estimation, gut samples were homogenized in PBS by grinding with a sterile plastic pestle and then centrifuged at 10,000 × *g* for 10 min at 4°C. The resultant supernatant was mixed 1:1 with Griess reagent (Sigma), and absorbance was measured at 595 nm using a plate reader (BioTek). Nitric oxide (NO) levels were calculated from a silver nitrite standard curve. For aconitase activity estimation, gut samples were homogenized in aconitase assay buffer and processed according to the manufacturer's instructions (MAK051-1KT; Sigma), and absorbance was measured at 450 nm. Aconitase activity levels were calculated from an isocitrate standard curve. Both NO and aconitase activity levels were represented relative to total protein content in each sample. Protein quantification was performed as described previously (55). The experiment was repeated three times.

Feeding rate. Ten *Drosophila* larvae from each line were infected with 10 symbiotic or axenic *Steinernema* nematodes or treated with sterile distilled water and then collected at 24 h postinfection. All larvae were fed on yeast paste containing 0.16% erioglaucine disodium salt (FD&C blue no. 1; Sigma) for 15 min. Larvae from each line were starved for 24 h and served as background controls. The protocol for spectrophotometric detection of the food dye has been described previously (56). Sample supernatants (200 μl each), obtained from centrifuging the larval homogenates, were loaded into a 96-well plate (Corning) and measured as the optical density at 633 nm (OD_{633}) using a plate reader (BioTek). The experiment was repeated three times.

Statistical analysis. For gene transcript level analysis, immunohistochemistry, nitric oxide estimation, and feeding rate, data analysis was performed using one-way analysis of variance (ANOVA) with a

Tukey *post hoc* test for multiple comparisons and an unpaired two-tailed *t* test. For survival experiments, a log rank (Mantel-Cox) and chi-square tests were performed. *P* values lower than 0.05 were considered statistically significant. All figures were generated using GraphPad Prism, version 7, software.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00094-19.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

We thank Kyle Devine and Sonali Gupta for maintaining the *Drosophila* stocks and members of the Department of Biological Sciences at The George Washington University for critical reading of the manuscript. We also thank Sneh Harsh for assistance with microscopy and imaging.

Research in the Eleftherianos lab is supported by the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. Kounatidis I, Ligoxygakis P. 2012. *Drosophila* as a model system to unravel the layers of innate immunity to infection. Open Biol 2:120075. https://doi.org/10.1098/rsob.120075.
2. Hetru C, Hoffmann JA. 2009. NF-κB in the immune response of *Drosophila*. Cold Spring Harb Perspect Biol 1:a000232. https://doi.org/10.1101/cshperspect.a000232.
3. Hoffmann JA, Reichhart JM. 2002. *Drosophila* innate immunity: an evolutionary perspective. Nat Immunol 3:121–126. https://doi.org/10.1038/ni0202-121.
4. Tanji T, Hu X, Weber AN, Ip YT. 2007. Toll and IMD pathways synergistically activate an innate immune response in *Drosophila* melanogaster. Mol Cell Biol 27:4578–4588. https://doi.org/10.1128/MCB.01814-06.
5. Arefin B, Kucerova L, Dobes P, Markus R, Strnad H, Wang Z, Hyrsl P, Zurovec M, Theopold U. 2014. Genome-wide transcriptional analysis of *Drosophila* larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. J Innate Immun 6:192–204. https://doi.org/10.1159/000353734.
6. Hallem EA, Rengarajan M, Ciche TA, Sternberg PW. 2007. Nematodes, bacteria, and flies: a tripartite model for nematode parasitism. Curr Biol 17:898–904. https://doi.org/10.1016/j.cub.2007.04.027.
7. Pena JM, Carrillo MA, Hallem EA. 2015. Variation in the susceptibility of *Drosophila* to different entomopathogenic nematodes. Infect Immun 83:1130–1138. https://doi.org/10.1128/IAI.02740-14.
8. Yadav S, Daugherty S, Shetty AC, Eleftherianos I. 2017. RNAseq analysis of the *Drosophila* response to the entomopathogenic nematode *Steinernema*. G3 (Bethesda) 7:1955–1967. https://doi.org/10.1534/g3.117.041004.
9. Castillo JC, Reynolds SE, Eleftherianos I. 2011. Insect immune responses to nematode parasites. Trends Parasitol 27:537–547. https://doi.org/10.1016/j.pt.2011.09.001.
10. Silverman N, Maniatis T. 2001. NF-κB signaling pathways in mammalian and insect innate immunity. Genes Dev 15:2321–2342. https://doi.org/10.1101/gad.909001.
11. Stock SP. 2005. Insect-parasitic nematodes: from lab curiosities to model organisms. J Invertebr Pathol 89:57–66. https://doi.org/10.1016/j.jip.2005.02.011.
12. Goodrich-Blair H. 2007. They've got a ticket to ride: *Xenorhabdus nematophila-Steinernema carpocapsae* symbiosis. Curr Opin Microbiol 10:225–230. https://doi.org/10.1016/j.mib.2007.05.006.
13. Ciche TA, Ensign JC. 2003. For the insect pathogen *Photorhabdus luminescens,* which end of a nematode is out? Appl Environ Microbiol 63:1890–1897. https://doi.org/10.1128/AEM.69.4.1890-1897.2003.
14. Martens EC, Goodrich-Blair H. 2005. The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. Cell Microbiol 7:1723–1735. https://doi.org/10.1111/j.1462-5822.2005.00585.x.
15. Goodrich-Blair H, Clarke DJ. 2007. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. Mol Microbiol 64:260–268. https://doi.org/10.1111/j.1365-2958.2007.05671.x.
16. Richards GR, Goodrich-Blair H. 2009. Masters of conquest and pillage: *Xenorhabdus nematophila* global regulators control transitions from virulence to nutrient acquisition. Cell Microbiol 11:1025–1033. https://doi.org/10.1111/j.1462-5822.2009.01322.x.
17. Yadav S, Shokal U, Forst S, Eleftherianos I. 2015. An improved method for generating axenic entomopathogenic nematodes. BMC Res Notes 8:461. https://doi.org/10.1186/s13104-015-1443-y.
18. Hyrsl P, Dobes P, Wang Z, Hauling T, Wilhelmsson C, Theopold U. 2011. Clotting factors and eicosanoids protect against nematode infections. J Innate Immun 3:65–70. https://doi.org/10.1159/000320634.
19. Kucerova L, Broz V, Arefin B, Maaroufi HO, Hurychova J, Strnad H, Zurovec M, Theopold U. 2016. The *Drosophila* chitinase-like protein IDGF3 is involved in protection against nematodes and in wound healing. J Innate Immun 8:199–210. https://doi.org/10.1159/000442351.
20. Yadav S, Eleftherianos I. 2018. The imaginal disc growth factors 2 and 3 participate in the *Drosophila* response to nematode infection. Parasite Immunol 40:e12581. https://doi.org/10.1111/pim.12581.
21. Carlson JR, Hogness DS. 1985. The *Jonah* genes: a new multigene family in *Drosophila melanogaster*. Dev Biol 108:341–354. https://doi.org/10.1016/0012-1606(85)90038-7.
22. Carlson JR, Hogness DS. 1985. Developmental and functional analysis of *Jonah* gene expression. Dev Biol 108:355–368. https://doi.org/10.1016/0012-1606(85)90039-9.
23. Hafen E, Levine M, Garber RL, Gehring WJ. 1983. An improved in situ hybridization method for the detection of cellular RNAs in *Drosophila* tissue sections and its application for localizing transcripts of the homeotic *Antennapedia* gene complex. EMBO J 2:617–623. https://doi.org/10.1002/j.1460-2075.1983.tb01472.x.
24. Akam ME, Carlson JR. 1985. The detection of *Jonah* gene transcripts in *Drosophila* by in situ hybridization. EMBO J 4:155–161. https://doi.org/10.1002/j.1460-2075.1985.tb02330.x.
25. Fernandez-Ayala DJ, Chen S, Kemppainen E, O'Dell KM, Jacobs HT. 2010. Gene expression in a *Drosophila* model of mitochondrial disease. PLoS One 5:e8549. https://doi.org/10.1371/journal.pone.0008549.
26. Carpenter J, Hutter S, Baines JF, Roller J, Saminadin-Peter SS, Parsch J, Jiggins FM. 2009. The transcriptional response of *Drosophila melanogaster* to infection with the sigma virus (*Rhabdoviridae*). PLoS One 4:e6838. https://doi.org/10.1371/journal.pone.0006838.
27. Chtarbanova S, Lamiable O, Lee KZ, Galiana D, Troxler L, Meignin C, Hetru C, Hoffmann JA, Daeffler L, Imler JL. 2014. *Drosophila* C virus systemic infection leads to intestinal obstruction. J Virol 88:14057–14069. https://doi.org/10.1128/JVI.02320-14.
28. Brun S, Vidal S, Spellman P, Takahashi K, Tricoire H, Lemaitre B. 2006. The MAPKKK Mekk1 regulates the expression of *Turandot* stress genes in response to septic injury in *Drosophila*. Genes Cells 11:397–407. https://doi.org/10.1111/j.1365-2443.2006.00953.x.
29. Imler JL, Bulet P. 2005. Antimicrobial peptides in *Drosophila*: structures, activities and gene regulation. Chem Immunol Allergy 86:1–21. https://doi.org/10.1159/000086648.
30. Kaneko T, Silverman N. 2005. Bacterial recognition and signalling by the

Drosophila IMD pathway. Cell Microbiol 7:461–469. https://doi.org/10.1111/j.1462-5822.2005.00504.x.
31. McEwen DG, Peifer M. 2005. *Puckered*, a *Drosophila* MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. Development 132:3935–3946. https://doi.org/10.1242/dev.01949.
32. Buchon N, Silverman N, Cherry S. 2014. Immunity in *Drosophila melanogaster*—from microbial recognition to whole-organism physiology. Nat Rev Immunol 14:796–810. https://doi.org/10.1038/nri3763.
33. Perochon J, Carroll LR, Cordero JB. 2018. Wnt signaling in intestinal stem cells: lessons from mice and flies. Genes (Basel) 4:E138. https://doi.org/10.3390/genes9030138.
34. Steinhart Z, Angers S. 2018. Wnt signaling in development and tissue homeostasis. Development 145:dev146589. https://doi.org/10.1242/dev.146589.
35. Liu X, Hodgson JJ, Buchon N. 2017. *Drosophila* as a model for homeostatic, antibacterial, and antiviral mechanisms in the gut. PLoS Pathog 13:e1006277. https://doi.org/10.1371/journal.ppat.1006277.
36. Aymeric JL, Givaudan A, Duvic B. 2010. *Imd* pathway is involved in the interaction of *Drosophila melanogaster* with the entomopathogenic bacteria, *Xenorhabdus nematophila* and *Photorhabdus luminescens*. Mol Immunol 47:2342–2348. https://doi.org/10.1016/j.molimm.2010.05.012.
37. Castillo JC, Shokal U, Eleftherianos I. 2013. Immune gene transcription in *Drosophila* adult flies infected by entomopathogenic nematodes and their mutualistic bacteria. J Insect Physiol 59:179–185. https://doi.org/10.1016/j.jinsphys.2012.08.003.
38. Shokal U, Eleftherianos I. 2017. Thioester-containing protein-4 regulates the *Drosophila* immune signaling and function against the pathogen *Photorhabdus*. J Innate Immun 9:83–93. https://doi.org/10.1159/000450610.
39. Lee WC, Beebe K, Sudmeier L, Micchelli CA. 2009. *Adenomatous polyposis coli* regulates *Drosophila* intestinal stem cell proliferation. Development 136:2255–2264. https://doi.org/10.1242/dev.035196.
40. Micchelli CA, Perrimon N. 2006. Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. Nature 439:475–479. https://doi.org/10.1038/nature04371.
41. Ohlstein B, Spradling A. 2006. The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells. Nature 439:470–474. https://doi.org/10.1038/nature04333.
42. Buchon N, Poidevin M, Kwon HM, Guillou A, Sottas V, Lee BL, Lemaitre B. 2009. A single modular serine protease integrates signals from pattern-recognition receptors upstream of the *Drosophila* Toll pathway. Proc Natl Acad Sci U S A 106:12442–12447. https://doi.org/10.1073/pnas.0901924106.
43. Bejsovec A. 2013. Wingless/Wnt signaling in *Drosophila*: the pattern and the pathway. Mol Reprod Dev 80:882–894. https://doi.org/10.1002/mrd.22228.
44. Choi NH, Kim JG, Yang DJ, Kim YS, Yoo MA. 2008. Age-related changes in *Drosophila* midgut are associated with PVF2, a PDGF/VEGF-like growth factor. Aging Cell 7:318–334. https://doi.org/10.1111/j.1474-9726.2008.00380.x.
45. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B. 2006. Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model. PLoS Pathog 2:e56. https://doi.org/10.1371/journal.ppat.0020056.
46. Xiang J, Bandura J, Zhang P, Jin Y, Reuter H, Edgar BA. 2017. EGFR-dependent TOR-independent endocycles support *Drosophila* gut epithelial regeneration. Nat Commun 8:15125. https://doi.org/10.1038/ncomms15125.
47. Toubarro D, Lucena-Robles M, Nascimento G, Santos R, Montiel R, Veríssimo P, Pires E, Faro C, Coelho AV, Simões N. 2010. Serine protease-mediated host invasion by the parasitic nematode *Steinernema carpocapsae*. J Biol Chem 285:30666–30675. https://doi.org/10.1074/jbc.M110.129346.
48. Carton Y, Frey F, Nappi AJ. 2009. Parasite-induced changes in nitric oxide levels in *Drosophila paramelanica*. J Parasitol 95:1134–1141. https://doi.org/10.1645/GE-2091.1.
49. Eleftherianos I, More K, Spivack S, Paulin E, Khojandi A, Shukla S. 2014. Nitric oxide levels regulate the immune response of *Drosophila melanogaster* reference laboratory strains to bacterial infections. Infect Immun 82:4169–4181. https://doi.org/10.1128/IAI.02318-14.
50. Wu SC, Liao CW, Pan RL, Juang JL. 2012. Infection-induced intestinal oxidative stress triggers organ-to-organ immunological communication in *Drosophila*. Cell Host Microbe 11:410–417. https://doi.org/10.1016/j.chom.2012.03.004.
51. Lemaitre B, Hoffmann J. 2007. The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25:697–743. https://doi.org/10.1146/annurev.immunol.25.022106.141615.
52. Le Bras S, Van Doren M. 2006. Development of the male germline stem cell niche in *Drosophila*. Dev Biol 294:92–103. https://doi.org/10.1016/j.ydbio.2006.02.030.
53. White GF. 1927. A method for obtaining infective nematode larvae from cultures. Science 66:302–303. https://doi.org/10.1126/science.66.1709.302-a.
54. Shokal U, Yadav S, Atri J, Accetta J, Kenney E, Banks K, Katakam A, Jaenike J, Eleftherianos I. 2016. Effects of co-occurring *Wolbachia* and *Spiroplasma* endosymbionts on the *Drosophila* immune response against insect pathogenic and non-pathogenic bacteria. BMC Microbiol 16:16. https://doi.org/10.1186/s12866-016-0634-6.
55. Yadav S, Frazer J, Banga A, Pruitt K, Harsh S, Jaenike J, Eleftherianos I. 2018. Endosymbiont-based immunity in *Drosophila melanogaster* against parasitic nematode infection. PLoS One 13:e0192183. https://doi.org/10.1371/journal.pone.0192183.
56. Kaun KR, Riedl CA, Chakaborty-Chatterjee M, Belay AT, Douglas SJ, Gibbs AG, Sokolowski MB. 2007. Natural variation in food acquisition mediated via a *Drosophila* cGMP-dependent protein kinase. J Exp Biol 210:3547–3558. https://doi.org/10.1242/jeb.006924.