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Balint Z. Kacsoh, University of Alabama at Birmingham
James A. Mobley, University of Alabama at Birmingham
Gregory J. Bowersock, University of Alabama at Birmingham
James Taylor, Emory University
Todd Schlenke, Emory University

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Parasitoid wasp venom SERCA regulates Drosophila calcium levels and inhibits cellular immunity

Nathan T. Mortimer,1 Jeremy Goecks,2,3 Balint Z. Kacsoh,4 James A. Mobley,3,5 Gregory J. Bowersock,3,5 James Taylor,2,3 and Todd A. Schlenke*

Departments of 1Biology and 2Mathematics and Computer Science, Emory University, Atlanta, GA 30322; and 3Department of Surgery and 4Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294

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Because parasite virulence factors target host immune responses, identification and functional characterization of these factors can provide insight into poorly understood host immune mechanisms. The fruit fly Drosophila melanogaster is a model system for understanding humoral innate immunity, but Drosophila cellular innate immune responses remain incompletely characterized. Fruit flies are regularly infected by parasitoid wasps in nature and, following infection, flies mount a cellular immune response culminating in the cellular encapsulation of the wasp egg. The mechanistic basis of this response is largely unknown, but wasps use a mixture of virulence proteins derived from the venom gland to suppress cellular encapsulation. To gain insight into the mechanisms underlying wasp virulence and fly cellular immunity, we used a joint transcriptomic/proteomic approach to identify venom genes from Ganaspis sp.1 (G1), a previously uncharacterized Drosophila parasitoid species, and found that G1 venom contains a highly abundant sarcoplasmic reticulum calcium ATPase (SERCA) pump. Accordingly, we found that fly immune cells termed plasmatocytes normally undergo a cytoplasmic calcium burst following infection, and that this calcium burst is required for activation of the cellular immune response. We further found that the plasmatocyte calcium burst is suppressed by G1 venom in a SERCA-dependent manner, leading to the failure of plasmatocytes to become activated and migrate toward G1 eggs. Finally, by genetically manipulating plasmatocyte calcium levels, we were able to alter fly immune success against G1 and other parasitoid species. Our characterization of parasitoid wasp venom proteins led us to identify plasmatocyte cytoplasmic calcium bursts as an important aspect of fly cellular immunity.

Results

In laboratory trials, we found that G1 readily attacks D. melanogaster larvae (Table S1), losing eggs that attach to internal fly tissues within 12 h post attack (PA) (Fig. 1A). G1 can efficiently escape encapsulation in D. melanogaster (Table S1), and following host pupation, the wasp eggs hatch into larvae that begin to consume host tissues (Fig. 1B), resulting in successful parasitization, with adult wasps emerging from nearly 100% of attacked D. melanogaster hosts (Fig. 1C). Drosophila parasitoid wasp species have widely differing host ranges (26), varying from specialists that target a narrow range of phylogenetically related species to generalists that can successfully infect a wide range of Drosophila species. To determine the host range of G1, we repeated our attack


The authors declare no conflict of interest.

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Data deposition: The G1 Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank (accession no. JQ808430). The version described in this paper is the first version, GAW01000000. G1 C0I and ITS sequences have been deposited in GenBank (accessions JQ808439, and JQ808440, respectively).

1To whom correspondence should be addressed. E-mail: nathantmortimer@gmail.com.

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We found that G1 Leptopilina heterotoma = tu(1)Sz phenotype Ca-P60A 0.01), (S2)(www.pnas.org/cgi/doi/10.1073/pnas.1222351110) mutants encapsulate their own posterior fat body mutant and Mortimer et al. (25). Larvae of G1 to suppress the self-encapsulation phenotype of To differentiate between these strategies, we tested the ability of immune suppressive, in which case the immune response is inhibited, or immune evasive, in which case the egg is not detected by the immune response, or immune evasive wasp would have no effect on the progression of self-encapsulation in tu(1)Sz mutants, whereas an immune suppressive wasp would be expected to inhibit the self-encapsulation phenotype. We found that the tu(1)Sz phenotype is significantly rescued by G1 attack (Fig. 1 D–F), demonstrating that G1 venom has immune suppressive properties.

Because immune suppression has been linked to the destruction of hemocytes, and in particular lamellocytes (25, 26), we assayed the total hemocyte count (THC) and the number of lamellocytes in G1 attacked larvae at 48 h PA. There is a significant increase in both THC (Fig. S1A) and lamellocyte number (Fig. S1B) in G1 attacked larvae compared with unattacked controls, and there was a significant decrease in THC and lamellocyte numbers relative to attacked by the avirulent wasp Leptopilina clavipes (which is encapsulated by D. melanogaster), demonstrating that G1-attacked larvae have a sufficient number of hemocytes for encapsulation of the wasp egg (Fig. S1). These data suggest that G1 venom suppresses the fly immune response by disabling, rather than destroying, host hemocytes.

To understand how G1 disables fly hemocytes, we used our recently described sequencing/bioinformatic approach to identify G1 venom genes (30). We first performed RNA-Seq on mRNAs isolated from dissected female wasp abdomens, and the sequence data were assembled into 234,516 transcripts (Table S2). The transcriptome was filtered by RSEM to remove low quality and low abundance sequences (30), resulting in a final assembly of 27,354 transcripts. We then used mass spectrometry to identify the proteins in purified venom, and the resulting 2,891 peptides were mapped against the transcriptome sequences. The peptides mapped to the predicted ORFs of 166 different transcripts (Datasets S1 and S2) for an average of 17.4 peptides per ORF, with an average protein coverage of 23.5% (Table S3). These venom genes accounted for just 0.61% of the expression-filtered assembly (Table S2), meaning that the identified venoms represent a specific subset of abdomen transcripts. We also found that the venom proteins are more likely to contain secretion signals than nonvenoms (28% of venom genes vs. 6% of nonvenoms; $P < 0.01$), consistent with the hypothesis that many venom genes encode small, classically secreted proteins (Table S4). Similar to Leptopilina boulardi and Leptopilina heterotoma venoms, Gene Ontology (GO) term enrichment suggests that G1 venom may regulate host physiology (via carbohydrate and nucleotide metabolism and antioxidant activity; Table S4) (30), but provides few clues to potential virulence mechanisms.

To further confirm the specificity of our approach, we performed suppression subtractive hybridization (SSH) (31) on cDNA samples made from G1 venom glands and carcasses (whole wasps with venom glands removed) to select for the most abundant transcripts that are specific to, or overrepresented in, the venom gland sample. Of the 71 SSH clones sequenced, 56 aligned to G1 transcripts and 14 of these clones matched our identified venom genes (Table 1), showing broad overlap across venom identification methods. Furthermore, we found that the most highly represented venom transcript by SSH was also one of the most abundant transcripts in the predicted ORFs of 166 different transcripts (Table S4), meaning that the identified venoms represent a specific subset of abdomen transcripts. We also found that the venom proteins are more likely to contain secretion signals than nonvenoms (28% of venom genes vs. 6% of nonvenoms; $P < 0.01$), consistent with the hypothesis that many venom genes encode small, classically secreted proteins (Table S4). Similar to Leptopilina boulardi and Leptopilina heterotoma venoms, Gene Ontology (GO) term enrichment suggests that G1 venom may regulate host physiology (via carbohydrate and nucleotide metabolism and antioxidant activity; Table S4) (30), but provides few clues to potential virulence mechanisms.

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Further, we found that the most highly represented venom transcript by SSH was also one of the most abundant transcripts in the predicted ORFs of 166 different transcripts (Table S4), meaning that the identified venoms represent a specific subset of abdomen transcripts. We also found that the venom proteins are more likely to contain secretion signals than nonvenoms (28% of venom genes vs. 6% of nonvenoms; $P < 0.01$), consistent with the hypothesis that many venom genes encode small, classically secreted proteins (Table S4). Similar to Leptopilina boulardi and Leptopilina heterotoma venoms, Gene Ontology (GO) term enrichment suggests that G1 venom may regulate host physiology (via carbohydrate and nucleotide metabolism and antioxidant activity; Table S4) (30), but provides few clues to potential virulence mechanisms.
Interestingly, only SERCA_{1002} was specifically identified by both venom mass spectrometry and SSH sequencing (Fig. S2), despite the presence of both transcripts in wasp abdomens. To test the idea that SERCA_{1002} represents a venom-specific isoform, we performed transcript-specific PCR on cDNA samples from venom glands and carcasses. We found SERCA_{1020} transcript in both carcass and venom gland samples, whereas SERCA_{1002} was specifically found in venom glands (Fig. 24). The identification of a single SERCA isoform by venom mass spectrometry despite the coexpression of both isoforms in the venom gland is consistent with SERCA_{1002} being a venom-specific isoform and supports the specificity of our results. To further confirm that SERCA is found in G1 venom, Western blots were performed on purified venom and total protein extract with anti-SERCA antiserum and we observed distinct bands in the two samples: smaller bands of ~150 kDa and a larger band of ~200 kDa that is excluded from the purified venom sample (Fig. 2B). These findings demonstrate that the SERCA found in our sequencing projects represents a true venom protein rather than a contaminant released from un-intentionally lysed cells during venom purification.

Table 1. Results of SSH sequencing

<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>Annotation</th>
<th>No. of SSH hits</th>
<th>Venom peptide hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>comp1045_seq1</td>
<td>SERCA</td>
<td>5</td>
<td>79</td>
</tr>
<tr>
<td>comp845_seq1</td>
<td>Troponin C</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>comp755_seq1</td>
<td>Arginine kinase</td>
<td>1</td>
<td>129</td>
</tr>
<tr>
<td>comp181_seq1</td>
<td>—</td>
<td>1</td>
<td>69</td>
</tr>
<tr>
<td>comp844_seq5</td>
<td>Neprilysin-2</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>comp630_seq1</td>
<td>Myosin LC alkali</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>comp171_seq2</td>
<td>Cysteine protease</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>comp317_seq1</td>
<td>—</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>comp636_seq2</td>
<td>Erythrose-4-P dehydrogenase</td>
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<td>4</td>
</tr>
<tr>
<td>comp1_seq1</td>
<td>28S rRNA</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>comp1999_seq1</td>
<td>—</td>
<td>4</td>
<td>—</td>
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<tr>
<td>comp50_seq1</td>
<td>18S rRNA</td>
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<td>—</td>
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<td>comp3562_seq1</td>
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<td>—</td>
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<tr>
<td>comp1032_seq1</td>
<td>CG31997</td>
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</table>

Identified transcripts are listed according to number of SSH clones, annotation, and peptide hits from venom proteomics.

To test whether venom SERCA is active, we designed an ex vivo assay to measure the ability of G1 venom to regulate intracellular calcium levels in fly cells. Purified G1 venom (or PBS control) was pretreated with either thapsigargin (TG), an irreversible and specific inhibitor of SERCA (33), or vehicle control (DMSO) and then dialyzed to remove excess TG. These samples were then incubated with plasmatocytes expressing a genetically encoded fluorescent calcium sensor (GCCaMP3; ref. 34) bled from third instar larvae. The intensity of GCCaMP3 fluorescence is proportional to intracellular calcium levels (34), and we assayed the ability of each sample to alter calcium levels by measuring GCCaMP3 fluorescence during the incubation period. We found that incubation with the PBS samples had no effect on GCCaMP3 fluorescence (Fig. 2C, blue and green lines). However, incubation with G1 venom/DMSO resulted in a significant decrease in GCCaMP3 fluorescence (Fig. 2C, yellow line), showing that G1 venom is able to manipulate host intracellular calcium levels. This effect of G1 venom was completely blocked by pretreatment with the SERCA inhibitor TG (Fig. 2C, red line), confirming that venom SERCA actively removes calcium ions from the plasmatocyte cytoplasm. The ability of venom SERCA to profoundly affect host plasmatocyte calcium...

Fig. 2. (A) Isoform-specific PCR of wasp carcass and venom gland cDNAs. (B) Western blot of wasp protein extracts and purified venom with anti-SERCA. (C) Corrected total cell fluorescence plotted over time during ex vivo incubation of GCCaMP3-expressing hemocytes with the indicated treatment. *P < 0.05 compared with PBS/DMSO control, error bars indicate SE, n = 3 independent replicates per treatment. (D) Isoform-specific PCR on cDNAs from D. melanogaster third instar larvae and hemocytes. (E and F) Corrected total cell fluorescence of GCCaMP3 and GFP expressing hemocytes, 6 h PA (E) and 24 h PA (F) with the indicated wasps, *P < 0.01 compared with respective unattacked controls, error bars indicate SE, n = 3 independent replicates for each time point per treatment.
levels suggests that it is functionally distinct from the endogenous fly plasmatocyte SERCA. Isoform-specific PCR shows that in contrast to the SERCA1β2 isoform found in G1 venom, fly hemocytes specifically express the SERCA1α2 isoform (Fig. 2D). Data from mammalian systems show that the two isoforms have different affinities for calcium and are subject to different modes of regulation, resulting in a higher maximal pumping rate for the shorter SERCA isoform (32, 35). If this difference is conserved in insect SERCA isoforms, it could account for the effect of G1 venom SERCA1α2 in fly hemocytes. These findings demonstrate that G1 venom antagonizes host hemocyte calcium levels in a SERCA-dependent manner.

The presence of active SERCA in G1 venom suggests that regulation of intracellular calcium concentration might be important for wasp virulence and, in turn, host resistance. To assay intracellular calcium levels in vivo, we expressed GCaMP3 in larval immune tissues with the Cg-GAL4 driver, which expresses in the plasmatocytes and fat body (36). We attacked these larvae with the avirulent wasp L. clavipes to assay calcium levels in a successful immune response and with G1 to assay the effect of G1 venom on host calcium levels in vivo. We simultaneously attacked larvae expressing calcium-independent GFP under the control of the Cg-GAL4 driver to control for differences in GAL4 expression following wasp attack. We found that plasmatocytes from L. clavipes–attacked larvae showed a significant increase in GCaMP3 fluorescence at 6 h PA, but no change in GFP fluorescence at this time (Fig. 2E), indicating a specific elevation of intracellular calcium levels following wasp attack. Following L. clavipes attack, we did not detect a change in GCaMP3 fluorescence either in plasmatocytes at 24 h PA (Fig. 2F) or in the fat body at either time point, demonstrating that the calcium burst is specific to plasmatocytes and is part of an immediate response to wasp attack. Conversely, plasmatocytes from G1–attacked larvae showed a significant decrease in GCaMP3 fluorescence at 6 h PA, whereas GFP levels remained constant (Fig. 2E). There was no alteration in GCaMP3 fluorescence in plasmatocytes at 24 h following G1 attack (Fig. 2F), or in the fat body at either time point. We also expressed GCaMP3 with the pan-hemocyte driver He-GAL4 (37) and did not detect GCaMP3 fluorescence in lamellocytes following attack by either wasp, confirming that the calcium burst is specific to plasmatocytes. These data show that the calcium regulatory activity of G1 venom demonstrated in our ex vivo assay is also functional in vivo following wasp attack and that G1 venom specifically targets the wasp-induced calcium burst in fly plasmatocytes that occurs immediately following attack.

The calcium burst seen in fly plasmatocytes following attack with the avirulent wasp L. clavipes suggests that calcium signaling may be required to activate hemocytes for encapsulation. A similar calcium burst is seen in both fly and mammalian immune cells in response to diverse pathogen stimuli (38, 39). To test for a role of the calcium burst in wasp egg encapsulation, we used He-GAL4 to express parvalbumin (PV), a vertebrate-specific calcium binding protein that negatively regulates calcium levels in D. melanogaster cells (40). At 72 h PA, there was no evidence of capsule formation in a large proportion of fly larvae attacked by the normally avirulent wasp L. clavipes (Fig. 3A), suggesting that He-GAL4–driven PV expression prevented capsule initiation by fly plasmatocytes. Furthermore, increased intracellular calcium is typically mediated by the release of calcium from ER stores by either the IP3 receptor (IP3R) or Ryanodine receptor (Ryr), the major ER calcium release channels in eukaryotic cells (41). Both of these calcium channels have homologs in D. melanogaster [encoded by the Inositol 1,4, 5-tris-phosphate receptor (Itp-r83A) and Ryanodine receptor 44F (Rya-r44F) genes, respectively] (41, 42), and we found that hemocyte-specific knockdown of Ryr-r44F, but not Itp-r83A, also resulted in a significant decrease in the proportion of L. clavipes eggs encapsulated by larval hemocytes (Fig. 3A). Ryr is also required for phagocytosis by fly hemocytes (38) and is important for the calcium burst in human B and T cells (39). These data show that the hemocyte calcium burst is important for hemocyte activation during the D. melanogaster encapsulation response and is conserved between mammalian and insect immune responses.

If the ability of G1 venom to antagonize the hemocyte calcium burst is important for G1 virulence, ectopically raising hemocyte calcium levels should allow fly larval hemocytes to encapsulate G1 eggs. To test this hypothesis, we used G1 wasps to attack larvae from two mutant lines with a demonstrated elevation of intracellular calcium levels (olf186-FY01467, the D. melanogaster homolog of the Orai calcium release-activated calcium channel, and Ca-P60A(Kum170)) (43, 44). We found that hemocytes from both olf186-FY01467 and Ca-P60A(Kum170) mutant larvae were able to encapsulate G1 eggs at a significantly higher rate than their genetic background controls [yellow,white (y,w) and Canton S (CS), respectively] (Fig. 3B and Table 2). Hemocyte-specific knockdown of Ca-P60A also conferred larvae with increased encapsulation ability against G1 (Fig. 3C), confirming the cell specificity of the calcium signaling phenotype. These results suggest that elevated calcium levels either block G1 virulence specifically or make fly larvae generally more wasp resistant. To distinguish between these possibilities, we attacked these same flies with the melanogaster subgroup specialist L. boulardi, a wasp whose venom does not contain homologs of any known calcium regulators (30). We found that L. boulardi eggs were not encapsulated by any of these genotypes regardless of calcium level (Table 2), showing that increased hemocyte intracellular calcium specifically affects G1 virulence.

![Graph](image)

**Fig. 3.** (A) Proportion of L. clavipes eggs encapsulated in the indicated genotypes. *P < 0.01 compared with control, error bars indicate SE in all graphs, within each graph, n = 3 independent replicates per genotype. (B) Proportion of G1 eggs encapsulated in the indicated genotypes. *P < 0.01 compared with genetic background controls. (C) Proportion of G1 eggs encapsulated in the indicated genotypes. *P < 0.01 compared with control.

Table 2. Encapsulation rates of G1 and L. boulardi eggs in the indicated genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Encapsulation rate of G1 eggs, %</th>
<th>n</th>
<th>Encapsulation rate of L. boulardi eggs, %</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>y,w</td>
<td>0</td>
<td>82</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>olf186-FY01467</td>
<td>21.7</td>
<td>92</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td>CS</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td>Ca-P60A(Kum170)</td>
<td>61.3</td>
<td>80</td>
<td>0</td>
<td>76</td>
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</table>


Discussion
The identification of wasp venom proteins led us to uncover a unique and important aspect of the Drosophila innate cellular immune response against macroparasites. Fly plasmatocytes undergo a cytoplasmic calcium burst within 6 h of parasitoid wasp infection, which is required for activation of the antiparasite immune response. A similar calcium burst is observed in plasmatocytes before phagocytosis of bacteria or apoptotic cells (38). When the calcium burst is genetically blocked by knockout or knockdown of Rya-R44F, plasmatocytes fail to initiate capsule formation in response to wasp infection (Fig. 3) and are unable to phagocytize invading bacteria (38), demonstrating that calcium signaling is a conserved mechanism among fly immune responses to various pathogens.

Calcium signaling also plays an important role in mammalian immunity. Increased cytoplasmic levels of calcium are observed in lymphocytes following activation of antigen receptors (39), and this increase activates the calcium-dependent phosphatase calcineurin, which is required for the activation of mammalian immune responses via regulation of the nuclear factor of activated T-cells (NFAT) family of transcriptional activators (40). Interestingly, calcineurin is also required in fly hemocytes for the activation of Imd pathway signaling in response to infection with Gram-negative bacteria (46), suggesting that the activation of diverse immune responses likely has a shared genetic basis. G1 wasps use venom SERCA to target this host-vasoactive plasmatocyte calcium burst and prevent the initiation of the encapsulation response. This finding demonstrates the advantage of studying naturally coevolving host-pathogen/parasite interactions to gain insight into conserved immune mechanisms.

Identifying parasitic wasp venom proteins can enhance our understanding of the delivery and function of immunomodulatory proteins in general. Wasp venom genes were proposed to encode small, classically secreted proteins (47), and this idea appears to be somewhat true of G1 venom proteins; 28% contained a predicted secretion signal, and these proteins had an average size of 44 kDa. However, venom SERCA is a large (110-kDa) transmembrane protein with no classical secretion signal sequence, and this finding was not unusual across G1 and other Hymenopteran venoms; G1 venom proteins range in size from 9 kDa to 379 kDa, and bioinformatic analyses using transmembrane domain prediction software (48) reveals that 7% (12/166) of G1 venom proteins and 20% (176/864) of the previously identified Hymenopteran venoms in GenBank (30) contain predicted transmembrane domains. How a parasite might secrete and deliver transmembrane proteins into hosts is unknown. The venom of parasitic wasps closely related to G1 have been shown to contain “virus-like particles,” thought to act as venom delivery vehicles, that enter host hemocytes to mediate wasp virulence (49), and we hypothesize that G1 SERCA may use a similar mechanism. Understanding the packaging of SERCA in G1 venom, and its delivery to host hemocytes, represents an interesting subject for future study.

Materials and Methods
Insect Strains. The following D. melanogaster strains were used in this study: the mutant alleles tu(1)Sz (29), Ca-P60A (40), and of1186 (40); the transgenic constructs G-GAL4 (36), He-GAL4 (37), UAS-GCaMP3 (34), UAS-GFP (42), UAS-PV (40), Ca-P60A (50), ttp-R83 (50), and Rya-R44F (40, 41); and the control lines y,w, Canton S (CS), Oregon R (OR), and w1118. For the host range experiment, the fly species used were as follows: Drosophila simulans, Drosophila mauritiana, Drosophila sechellia, Drosophila yakuba, Drosophila erecta, Drosophila ficaria, Drosophila ananassae, Drosophila pseudobscura, Drosophila pseudoobscura,wilsoni, Drosophila funebris, Drosophila immigrans, Drosophila mojavensis, Drosophila para melanica, and Drosophila virilis.

This study also used the following wasps: G1, L. clavipes (strain LcNet), and L. boulardi (strain LB17). The G1 wasps used in this study were caught in Homestead, FL, in 2008. G1 COI and ITS2 sequences have been deposited in GenBank (accession nos. JQ088430 and JQ088406, respectively). LcNet was provided by J. van Alphen (University of Amsterdam, Amsterdam) and LB17 has been described (28). Laboratory cultures of G1 and LB17 are maintained on D. melanogaster and LcNet is maintained on D. virilis.

Wasp Attack. Wasp attacks were performed as described (17). Briefly, three female wasps were allowed to attack 40 second instar fly larvae in 35-mm Petri dishes filled with Drosophila medium for a 72-h period at 25 °C. To assay attack and encapsulation rates, larvae were dissected and scored for the presence of an encapsulated wasp egg or live wasp larva. To assay elicitation rates, 30 fly larvae were recovered from each plate and allowed to eclose at 25 °C. The total number of flies and wasps that eclosed were determined 15 d and 30 d after infection, respectively, times by which all viable flies and wasps should have emerged. All experiments were performed in triplicate.

tut/1Sz Phenotype Suppression. The temperature-sensitive tut(1)Sz2 self-encapsulation mutant was used to assay wasp virulence strategy (29). The flies were raised at 28 °C and assayed for the ability of wasp venom to suppress the phenotype, 40 second instar larvae were attacked by three female wasps for 3 h at 28 °C. The attacked larvae were raised for a further 96 h at 28 °C. Attacked pupae and age-matched controls were then scored for the tut(1)Sz phenotype and dissected to ensure attack status; unattacked pupae were discarded from analysis.

Imaging. Images were acquired by using a Leica stereo-dissecting scope with a Moticam MIP 2.0 and Multi-Focus Pro software. Figures were compiled by using Adobe Photoshop.

Hemocyte Counts. Hemocyte counts were performed in triplicate according to ref. 17. After a 72-h wasp attack period, five larvae from each replicate were washed in Drosophila Ringer’s solution and bled into PBS containing 0.01% phenylthiourea to prevent melanization. Hemocytes were applied to a disposable hemocytometer (Incyto C-Chip DHC-N01) and allowed to adhere for 30 min. Hemocytes of each replicate were counted from 16.025 × 0.25 × 0.1 mm squares, and the counts were normalized to a per larva value.

Wasp Transcriptsomes. RNA was extracted from ~200 female wasp abdomens by using the standard TRIzol (Invitrogen) protocol. Poly(A)(RNAs were purified by using the Dynabeads mRNA Direct kit (Invitrogen) according to manufacturer specifications. Double-stranded cDNAs were synthesized by using the SuperScript II ds cDNA Synthesis kit (Invitrogen). The cDNAs were then sequenced by using an Illumina HiSeq 2000. Transcripts were de novo assembled by using Trinity (version 2011-10-29) (50) and filtered by RSEM (51) using a cutoff of one transcript per million. See ref. 30 for more detailed protocols. Tools for sequence signal analysis and GO term annotation and enrichment are described in ref. 30.

Mass Spectrometry. Venom proteins were purified from venom glands dissected into PBS supplemented with 0.5 mM EDTA and Complete Protease Inhibitor Mixture (Roche). Venom glands were homogenized under nonlysing conditions, and gland cells were pelleted by centrifugation. Venom proteins were run on SDS/PAGE, trypsinized, and subjected to Nano LC-MS/MS (52). The identified peptides were mapped back to the transcriptome data by using SEQUEST software. See ref. 30 for more detailed protocols.

SERCA Isoform-Specific PCRs. To assay expression of SERCA isoforms transcripts, total RNA was made from venom gland tissues or wasp carcasses (wasps with venom gland removed), and whole third instar larvae or dissected larval hemocytes, by using standard TRizol preparations as described above. RNA was reverse transcribed to cDNA by using the QuantiTect reverse transcriptase kit (Qiagen). Isoform specific primers were used to amplify SERCA from each cDNA sample (primer sequences available upon request).

SSH. SSH was performed essentially as described (31) by using venom gland cDNAs as the tester library and wasp carcass cDNAs as the driver library and with the following alterations: libraries were hybridized at a 30:1 (driver: tester) ratio and then used undiluted for two rounds of PCR amplification. PCR products were cloned into IPCR cloning kit (Qiagen). Isoform specific primers were used to amplify SERCA from each cDNA sample (primer sequences available upon request).

GCaMP3 Calcium Assays. Ex vivo assay. We used thapsigargin, a plant-derived sesquiterpene lactone that acts as a specific and irreversible inhibitor of SERCA (33). G1 venom was purified as described above and incubated with 1 μM thapsigargin in DM5O or DM5O control at room temperature for 15 min. Samples were then twice dialyzed against PBS at room temperature (15 min, 1 h), added to dissected GCaMP3-expressing third instar larval hemocytes,
and incubated at room temperature. During the incubation period, cells were imaged by using an Olympus BX51 microscope with a FITC filter and Olympus DP2-BSW software. Corrected total cell fluorescence (CTCF) was calculated as described (S3).

In vivo assay. Larvae expressing GCaMP3 or GFP were attacked by wasps as described and dissected at 6 or 24 h PA. Cells were imaged, and CTCFs were calculated as for the ex vivo assay.

**Statistics.** All analyses were performed in R version 2.15.0. Fisher’s exact test was used to compare tu/tu+3jz phenotype between G1 attacked and control samples and to compare the results of Signal P analysis on G1 venom and nonvenom genes. Total hemocyte counts and lamellocyte numbers were compared by ANOVA, and Tukey’s HSD test was used for pairwise comparisons. Differences in in vivo GCaMP3 and GFP fluorescence levels were compared by two-way ANOVA at 6 h and 24 h PA to test effects for of fly genotype and wasp attack. Tukey’s HSD test was used for pairwise comparisons to test the effect of venom and PBS on GCaMP3 fluorescence throughout the incubation period in the ex vivo assay.

we used repeated measures ANOVA (for the car R library) and pairwise tests to compare fluorescence at each time point. Finally, generalized linear models with binomial errors and logit link functions were used to examine differences in technical replicates and variations in use.