Pathogenesis of Listeria-Infected Drosophila wntD Mutants Is Associated with Elevated Levels of the Novel Immunity Gene edin

Michael D. Gordon1*, Janelle S. Ayres2, David S. Schneider2*, Roel Nusse1*

1 Department of Developmental Biology, Howard Hughes Medical Institute, Beckman Center, Stanford University School of Medicine, Stanford, California, United States of America, 2Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California, United States of America

Abstract

Drosophila melanogaster mount an effective innate immune response against invading microorganisms, but can eventually succumb to persistent pathogenic infections. Understanding of this pathogenesis is limited, but it appears that host factors, induced by microbes, can have a direct cost to the host organism. Mutations in wntD cause susceptibility to Listeria monocytogenes infection, apparently through the derepression of Toll-Dorsal target genes, some of which are deleterious to survival. Here, we use gene expression profiling to identify genes that may mediate the observed susceptibility of wntD mutants to lethal infection. These genes include the TNF family member eiger and the novel immunity gene edin (elevated during infection; synonym CG32185), both of which are more strongly induced by infection of wntD mutants compared to controls. edin is also expressed more highly during infection of wild-type flies with wild-type Salmonella typhimurium than with a less pathogenic mutant strain, and its expression is regulated in part by the Imd pathway. Furthermore, overexpression of edin can induce age-dependent lethality, while loss of function in edin renders flies more susceptible to Listeria infection. These results are consistent with a model in which the regulation of host factors, including edin, must be tightly controlled to avoid the detrimental consequences of having too much or too little activity.

Citation: Gordon MD, Ayres JS, Schneider DS, Nusse R (2008) Pathogenesis of Listeria-Infected Drosophila wntD Mutants Is Associated with Elevated Levels of the Novel Immunity Gene edin. PLoS Pathog 4(7): e1000111. doi:10.1371/journal.ppat.1000111

Editor: Frederick M. Ausubel, Massachusetts General Hospital, United States of America

Received September 7, 2006; Accepted June 26, 2008; Published July 25, 2008

Copyright: © 2008 Gordon et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: MDG was supported by a Howard Hughes Medical Institute predoctoral fellowship and a Stanford Graduate Fellowship. This work was supported by grants from the NIH to RN and DSS and by the Howard Hughes Medical Institute.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: dschneider@stanford.edu (DSS); rnusse@stanford.edu (RN)

† Current address: Department of Molecular and Cell Biology, Helen Wills Neuroscience Institute, University of California Berkeley, Berkeley, California, United States of America

Introduction

Drosophila has an effective innate immune system to combat infection. This response relies heavily on the Toll and Immune deficiency (Imd) pathways, both of which utilize NF-kB related transcription factors as central mediators of signaling: Dorsal and Dorsal-related immunity factor (Dif) in the case of Toll, and Relish (Rel) in the case of Imd (reviewed in [1–3]).

The Toll and Imd pathways have largely been characterized with respect to their role in the humoral immune response, a branch of immunity that is triggered through recognition of microbial molecular signatures by upstream components of both the Imd and Toll pathways and subsequent nuclear translocation and activation of the cognate NF-kB factor(s). The activation of these transcription factors leads to transcription of hundreds of genes following infection [4–6]. The most studied are the antimicrobial peptide (AMP) genes, which are transcribed in the fat body, leading to secretion of these peptides into the circulating hemolymph (reviewed in [7]).

In addition to its role in AMP regulation, the Toll pathway is also known to participate in two other branches of immunity: the deposition of melanin and the cellular immune response [8–12]. The cellular response in particular has become of increasing interest, as studies of Drosophila immunity progress beyond the characterization of acute responses to non-pathogenic bacteria to those involving chronic infections that eventually kill the fly [13–16]. Many of these model infections proceed intracellularly within the phagocytic cells of the circulating hemolymph, thereby shielding the bacteria from the action of circulating AMPs. This provides a convenient model system for studying the molecular interactions between pathogens and their hosts, including the processes that eventually lead to the host’s demise.

One principle that has been understood in mammals for decades, and seems to also be true in Drosophila, is that an immune response can be both beneficial and detrimental to a host. Indeed, the same signals that are critical to containing a localized infection will kill the host if uncontrolled [17]. One such signal is Tumor Necrosis Factor (TNF), which is both necessary to fight local infections of many organisms and sufficient to induce lethal septic shock if released systemically [18,19]. Homologous processes may also occur in Drosophila; loss of function mutations in the TNF family member eiger result in prolonged survival during infection with Salmonella typhimurium [14,20]. Thus Drosophila offers an appealing genetic system to uncover host genes that may have dual effects during the immune response, mediating deleterious consequences to both the pathogen and the host itself.

Previously, we reported evidence that flies mutant for the Wnt family member wntD have a defective immune system and
Author Summary

Like any organism, fruit flies respond to invading microorganisms by mounting an immune defense. Many aspects of the immune defense in fruit flies are similar to the inflammatory response in mammals, including the harmful effects of a sustained response against persistent pathogenic infections. We found in the past that mutations in the gene wntD cause flies to succumb more easily to Listeria monocytogenes infections, apparently by losing an element of control over the inflammatory response. How does the wntD gene work? In this paper, we have identified genes that may mediate the susceptibility of wntD mutants to lethal infection. These genes include edin, a homolog of the mammalian TNF gene, and a previously uncharacterized gene called edin (elevated during infection). Edin is expressed excessively in wntD mutant flies, and its expression also correlates with the level of pathogenesis induced by two different strains of Salmonella typhimurium. In its own right, overexpression of the edin gene can induce lethality, while losing edin function renders flies more susceptible to Listeria infection.

Our results support a model in which the regulation of host factors, including edin, must be tightly controlled to avoid the detrimental consequences of having too much or too little activity.

**Results**

wntD mutants exhibit upregulation of specific immune targets in the absence of infection

In order to gain insight into the processes that are misregulated in wntD mutants and that may contribute to their susceptibility to L. monocytogenes infection, we collected RNA from wntD and control flies under two conditions: naïve and 24 hours following infection with L. monocytogenes. We examine two groups of candidate mediators of the decreased survival of wntD mutants, and provide evidence that one of those genes, edin (elevated during infection; synonym CG32185), could be a novel effector of pathogenesis.

Cluster analysis reveals two groups of candidate mediators of wntD lethality

To identify genes as candidate mediators of wntD mutants’ infection sensitivity, cluster analysis was used [25]. Hierarchical clustering revealed several distinct groups of genes that showed controls. As shown in Figure 1, the top thirteen genes most induced by infection all showed higher levels of expression in uninfected wntD mutants compared to uninfected controls. Of these thirteen genes, seven showed an average of greater than 2-fold difference between mutants and controls and had p-values less than 0.025 (Figure 1 and Table 1). This set of genes was comprised of the novel immune gene edin, IME23, AttD, AttB, AttA, DipdB, and Dof, all of which are known to be induced by infection under various conditions [5,23,24]. It is worthwhile noting, however, that several known immune-regulated genes that were strongly induced by infection in our study showed no significant difference between wntD mutants and controls, including CG6639, CecB, TotM and Dmu (Figure 1 and data not shown).

Overall, the correlation coefficient (r) for these data sets was 0.14, with a p-value<0.0001. Calculating the coefficient of determination \( r^2 \) suggests that approximately 2% of the variation within the data can be explained by the correlation between the two data sets. This corresponds to approximately 235 genes, a plausible number given previous studies have indicated that about 400 genes are significantly regulated by infection [4]. In a similar analysis looking at the misregulation of immune genes in wntD mutants following infection, no significant correlation was observed (data not shown). As is evident from the cluster analysis presented below and the data in Table 1, a subset of immune-induced genes were expressed more highly in wntD mutants following infection, but many of the most highly induced immunity genes were not significantly different between wntD mutants and controls, and some were expressed at lower levels in the mutants. This may have resulted from a lack of sensitivity from the array at these high levels of expression, saturation of the signaling processes leading to induction of expression, or dominant negative effects of activated Dorsal on the activity of other NF-kB proteins.

Cluster analysis reveals two groups of candidate mediators of wntD lethality

Unlike almost any organism, fruit flies respond to invading microorganisms by mounting an immune defense. Many aspects of the immune defense in fruit flies are similar to the inflammatory response in mammals, including the harmful effects of a sustained response against persistent pathogenic infections. We found in the past that mutations in the gene wntD cause flies to succumb more easily to Listeria monocytogenes infections, apparently by losing an element of control over the inflammatory response. How does the wntD gene work? In this paper, we have identified genes that may mediate the susceptibility of wntD mutants to lethal infection. These genes include edin, a homolog of the mammalian TNF gene, and a previously uncharacterized gene called edin (elevated during infection). Edin is expressed excessively in wntD mutant flies, and its expression also correlates with the level of pathogenesis induced by two different strains of Salmonella typhimurium. In its own right, overexpression of the edin gene can induce lethality, while losing edin function renders flies more susceptible to Listeria infection.

Our results support a model in which the regulation of host factors, including edin, must be tightly controlled to avoid the detrimental consequences of having too much or too little activity.

Cluster analysis reveals two groups of candidate mediators of wntD lethality

To identify genes as candidate mediators of wntD mutants’ infection sensitivity, cluster analysis was used [25]. Hierarchical clustering revealed several distinct groups of genes that showed
correlation in their expression patterns across the four different conditions. However, two related clusters of genes were selected for further analysis based on the following rationale: the expression of genes actively contributing to pathogenesis will most likely be elevated following infection, and genes within this group that might be implicated in the more rapid lethality seen in wntD mutants would be expressed higher in these mutants. The average expression level under each condition for the two selected clusters (Clusters A and B) are shown in Figure 2. The clusters differ in that Cluster A shows a greater overall change in response to infection than does Cluster B (Figure 2).

Cluster A includes a number of known targets of infection, including several AMPs (Table S1). While it is certainly possible that several of these are contributing to pathogenesis in the fly, one uncharacterized gene in particular stood out based on its levels of expression. Confirmed by quantitative RT-PCR, edin shows strong induction by Listeria infection (45 fold), and dramatically higher levels of expression in infected wntD mutants versus infected controls (7.5 fold) (Figure 2B). Furthermore, only a 1.7 fold difference was seen between mutants and controls prior to infection, illustrating synergy between Listeria infection and the absence of wntD function on the regulation of edin.

Table 1. List of top 13 genes most induced by infection of wild-type flies.

| Gene     | WT infected/WT uninfected t-test | wntD uninfected/WT uninfected t-test | wntD infected/WT infected t-test |
|----------|----------------------------------|-------------------------------------|----------------------------------|
| Cecropin C | 652.54 0.003                      | 12.16 0.2                           | 0.70 0.01                        |
| edin      | 394.73 0.008                      | 15.36 0.02                         | 5.14 0.0001                      |
| M23       | 166.01 0.04                       | 10.14 0.0009                      | 0.98 0.9002                      |
| CG6639    | 131.72 0.002                      | 1.15 0.8                            | 0.21 0.0004                      |
| Diptericin B | 90.56 0.005                   | 4.36 0.02                          | 1.04 0.004                       |
| Attacin D | 88.19 0.0004                     | 3.65 0.008                         | 1.87 0.004                       |
| Attacin B | 57.37 0.0009                     | 3.85 0.003                         | 1.04 0.003                       |
| Attacin A | 55.71 0.007                      | 4.70 0.02                          | 1.67 0.004                       |
| Defensin  | 44.85 0.001                      | 2.92 0.008                         | 1.30 0.01                        |
| Turandot M | 41.93 0.03                       | 1.40 0.5                            | 1.68 0.04                        |
| CG30098   | 33.55 0.01                       | 1.33 0.08                          | 1.55 0.2                         |
| Cecropin B | 23.08 0.01                    | 1.21 0.2                            | 0.97 0.8                         |
| Attacin C | 20.70 0.008                      | 1.90 0.09                          | 1.09 0.4                         |

"WT infected/WT uninfected" shows the induction of each gene by infection of wild-type flies with L. monocytogenes. "wntD uninfected/WT uninfected" shows the enrichment of each gene in wntD mutants prior to infection. "wntD infected/WT infected" shows the enrichment of each gene in wntD mutants following infection. t-test columns indicate the p-value for the comparison given in the leftward column.

doi:10.1371/journal.ppat.1000111.t001

Figure 2. Cluster analysis identifies candidates for genes involved in increased mortality of wntD mutants. (A) Graph illustrating average values for genes in clusters A and B under each of the four conditions tested. Solid line indicates Cluster A, dashed line indicates Cluster B. Each data point is the mean of all three replicates of all genes in the cluster (B) Normalized Quantitative RT-PCR data for expression of edin under each condition. edin shows increased expression upon infection, and is significantly elevated in wntD mutants following infection. (C) Normalized Quantitative RT-PCR data for expression of eiger under conditions each condition. eiger expression is changed only in wntD mutants following infection. Expression levels are normalized to Ribosomal protein 15a, and the value of the control uninfected sample is set to 1. Error bars indicate s.e.m. Asterisks indicate significance by student t-test: ** = p < 0.01.

doi:10.1371/journal.ppat.1000111.g002
Cluster B is composed of genes that show less dramatic changes in response to infection, but are still elevated in wntD mutants versus controls (Figure 2A, Table S2). It seems likely that this set of genes would include those that are regulated by processes aside from those sensing acute infection (Toll, Imd), and may include both mediators and markers of pathogenesis. Interestingly, this cluster includes the gene eiger, a TNF homolog known to mediate disease processes following Salmonella and Mycobacterium infections [14,20]. In this case, using quantitative RT-PCR, we see a statistically significant elevation of eiger expression only in infected wntD mutants (Figure 2C).

Edin encodes a novel protein that is misregulated in wntD mutants

The edin gene is predicted to encode a secreted protein 115 amino acids in length (http://flybase.bio.indiana.edu/bin/fbadg.html?FBgn0052185). The gene has homologs in other insects, but not in other Phyla. (Figure 3). Furthermore, no known conserved domains were identified in Edin or its putative ortholog in Drosophila pseudobscura and secondary structure prediction failed to identify any similar proteins or motifs based folding patterns (data not shown).

To answer the question of whether edin misregulation in wntD mutants is specific to infection with Listeria, wntD and control flies were injected with the non-pathogenic gram-positive bacteria Micrococcus luteus. Analysis of Edin expression levels prior to and following infection were monitored using quantitative RT-PCR (Figure 4A). The results are strikingly similar to those seen for Listeria infection; expression of edin is elevated 1.7-fold in wntD mutants compared to controls prior to infection, and 8-fold following infection. Again, a synergistic relationship is seen between infection and the presence of the wntD mutation. The smaller magnitude of edin induction seen in response to M. luteus compared to Listeria (~10 fold versus ~45 fold in wild-type flies) may be explained by the shorter time course of infection (5 hours versus 24 hours), a smaller bacterial load at the time of assay, or intrinsic differences between the two species of bacteria.

The strong regulation of edin in response to bacterial challenge raises the question of whether its transcription is regulated by the Toll and/or Imd pathways. To investigate this possibility, the induction of edin was monitored in genetic backgrounds each containing a loss of function mutation for a component in one of the pathways (Figure 4B). Mutations in imd reduced the expression of edin following infection to approximately 25% of that seen in wild type. This indicates that the Imd pathway participates in edin regulation, but is not strictly required for its induction following infection. By contrast, loss of function mutations in the Toll ligand spatzle did not reduce the transcriptional induction of edin, and in fact resulted in higher than normal levels of expression. This has been seen for other genes (such as dpericin) that do not have a strong requirement for Toll signaling, and could be due to increased survival of the bacteria in these mutants (data not shown; [4]). Levels of edin were slightly elevated (4-fold) in naive flies carrying a dominantly activated allele of Toll in the absence of infection (Tollac, Figure 4B). These data indicate that Toll signaling may be sufficient to induce low levels of edin expression, but is not required for its expression.

Edin is required to fight Listeria infections

In order to investigate whether Edin plays an essential role in disease progression, we knocked down its expression using two independently made UAS-driven RNA interference (RNAi) constructs. Edin expression was knocked down using the fat body driver Lsp2-Gal4 to ablate its activity in a major immune tissue. Edin knockdown flies displayed increased sensitivity to Listeria monocytogenes, with flies dying significantly faster than all controls (p<0.001) (Figure 5). This demonstrates that edin is required for an effective host response against Listeria infection. Interestingly, bacterial loads in edin knockdown flies were not significantly different from controls (data not shown). This places edin among several previously identified genes that affect a fly’s endurance during Listeria infection rather than its ability to combat bacterial growth [26]. While the mechanism for this effect is unknown, we hypothesize that knockdown of edin expression alters the physiology of the fly in a way that makes it more susceptible to Listeria pathogenesis.

Detrimental effects of Edin misregulation

Immunity can be a double-edged sword that has to be regulated precisely to help defend against infection while limiting damage to the body. Overexpression of genes misregulated during an immune response led us to edin and we found that it is required for fly survival during an L.monocytogenes infection. Next, we thought it was of great interest to determine whether Edin expression contributed to pathology. We first looked for more evidence that Edin was associated with pathology under different circumstances. We compared the expression of edin following infection of wild-type flies with wild-type Salmonella typhimurium or a SPI1, SPI2 mutant strain of Salmonella that has decreased pathogenicity [14]. As shown in Figure 4C, edin was expressed at significantly higher levels during the course of a wild-type Salmonella infection compared to the less pathogenic strain at both time points tested. The more dramatic difference was seen later in infection, when edin transcript levels were over 5-fold higher in flies infected with wild-type Salmonella (Figure 4C). These data add more correlative evidence that Edin is associated with pathogenesis.

Do edin expression levels affect survival? To answer this question, we first overexpressed edin using the UAS-Gal4 system. Two different insertions of the p-element carrying UAS-edin resulted in varied levels of expression, with one insert (19-3) causing expression levels ~10 fold over wild type when combined with actin-Gal4, and the other overexpressing edin over 500 fold

**Figure 3. Sequence alignment of Edin with identified homologs.** Alignment of three insect homologs identified by BLAST search: Drosophila melanogaster edin, Drosophila pseudobscura GA16743-PA, and Stomoxys calcitrans (stable fly) EST (NCBI accession D952940). doi:10.1371/journal.ppat.1000111.g003
We observed that the higher level of expression resulted in significant levels of lethality prior to and following eclosion (Figure 6B,C). Flies strongly overexpressing *edin* survived to adulthood at a frequency less than 50% of expected, compared to 111% for the lower expresser. The value greater than 100% can most likely be attributed to non-specific deleterious effects of carrying the CyO balancer. The average lifespan of those flies surviving to adulthood was also significantly reduced in the context of strong overexpression of *edin* (Figure 6C). Given that *wntD* mutants infected with *L. monocytogenes* displayed similar levels of

![Figure 4](Image)

**Figure 4. Edin expression is partly regulated by the Imd pathway, and is correlated with increased *S. typhimurium* pathogenesis.** (A) Quantitative RT-PCR data for expression of *edin* in *yw; wntD* and *yw* control flies prior to and following infection with *M. luteus*. Expression is induced by infection with *M. luteus*, and expression is significantly elevated in *wntD* mutants following infection. (B) Quantitative RT-PCR data for expression of *edin* following infection with a mixture of gram-positive and gram-negative bacteria in various host genetic backgrounds. Induction is mitigated in *imd*10191 mutants, demonstrating input from the Imd pathway in controlling the expression of *edin*. Flies of the genotype spz*+/spz* express *edin* at higher levels than controls. Uninfected T1016/+ flies show mild induction of *edin* in the absence of infection (4.2 fold) (ND = this genotype was not assayed following infection). (C) Quantitative RT-PCR data for expression of *edin* in wild-type flies following infection with a wild-type strain of *Salmonella typhimurium* (SL1344) or a strain mutant for SPI1 and SPI2 (BJ66/P3F4). Values are relative to those in uninfected wild-type flies. Expression levels in all cases are normalized to Ribosomal protein 15a. Error bars indicate s.e.m. Asterisks indicate significance by student t-test: * = p < 0.05, ** = p < 0.01.

doi:10.1371/journal.ppat.1000111.g004

![Figure 5](Image)

**Figure 5. Knockdown of *edin* expression sensitizes flies to *Listeria* infection.** Survival curves shown for two independent UAS-RNAi lines against *edin* controlled by the fat body driver Lsp2-Gal4. All heterozygous controls were created by mating to w*1118*, and +/+ denotes w*1118*. Edin knockdowns are significantly different from all three controls by Log Rank test (p < 0.001). Significant differences between Listeria challenged *edin* knockdown and control flies were seen in two additional repetitions of this experiment. All experiments tested 60 flies per condition.

doi:10.1371/journal.ppat.1000111.g005
expression to the strong insertion of UAS-edin (about 350 fold over uninfected wild-type flies; Figure 2B), it is possible that edin expression is contributing to the rapid mortality of these mutants.

Taken together with the observation that edin loss of function mutants show increased sensitivity to L. monocytogenes, these data support a model in which edin expression must be tightly controlled during a host response to infection: moderate induction is essential to an effective response, but uncontrolled, high levels of expression become detrimental to the host animal.

Discussion

The idea that an elevated immune response could be detrimental to an infected host is at first unintuitive. However, it is well established that, like most other biological processes, proper regulation and containment of the immune response is critical to an animal’s viability. In mammals, LPS-triggered TNF release at a site of injury/infection is critical to mobilize the immune and inflammatory processes required to fight the infection, but in the rare cases when this reaction becomes uncontrolled and systemic, the shock will rapidly kill the host [17]. Studies in the fly have shown that genetic removal of a TNF-like molecule called Eiger increases flies’ longevity during some infections, but decreases it during others [14,20]. Thus eiger appears to be a double-edged sword – necessary for fighting some infections, but not without a cost to the host. Similarly, flies carrying T10th mutations, which dominantly activate the Toll pathway, die more rapidly from Drosophila X virus infection, despite lower viral loads [27,28], and over-activation of the IMD pathway has a negative impact on larval survival during bacterial infection [28]. These results imply that both the Toll and IMD pathways activate the transcription of genes that have a deleterious effect on a fly’s survival during pathogenic infection, one of which could well be eiger. In light of these findings, the observation that wntD mutants die more quickly from Listeria infection, while hyperactivating immune genes, is less surprising. Furthermore, this phenotype is suppressed by loss of dorsal, implying that Dorsal is actively regulating processes that decrease the fly’s survival [21].

Edin as a candidate mediator of pathogenesis

We presented two experiments that compared the expression profiles of flies undergoing two different levels of pathogenesis: wntD versus control flies following L. monocytogenes infection, and wild-type S. typhimurium versus a SPI1, SPI2 mutant strain. In both cases the gene edin was strongly elevated in the flies closer to death. In comparing wntD mutant versus control flies following Listeria infection, RNA samples were taken 1 day after infection, shortly before the mutants exhibit a sharp decrease in survival [21]. Expression of edin was about 8-fold higher in the wntD mutants.

Similarly, at 7 days post Salmonella infection, flies infected with wild type have begun to die, while those infected with a SPI1, SPI2 mutant strain will live for several more days despite carrying dramatically higher loads of bacteria [14]. In this case, we observed a 5-fold elevation in edin expression in the flies beginning to die. Thus, high edin expression is correlated with increased pathogenesis, although a causal relationship is not established by these data.

Two results strongly suggest that edin induction is not downstream of pathogenesis. First, edin expression is elevated following infection with M. luteus, a non-pathogenic bacterium,
and is more strongly induced in *wntD* mutants (Figure 2A). These data demonstrate that pathogenesis is not required for *edin* expression. Second, the Imd pathway appears to play a significant role in regulating *edin*, and this pathway is acutely induced upon recognition of bacterial moieties and does not strictly depend on pathogenesis [29–31].

Could Edin play a causal role upstream of pathogenesis? The induction of *edin* during *M. luteus* infection without any demonstrable pathogenesis suggests that the amount of Edin produced during this infection is not sufficient to elicit pathogenesis. However, these levels are approximately 5-fold lower than those seen for *Listeria* infection and persist for less than a day (data not shown), in contrast to the chronic induction during infection with *Listeria* or *Salmonella*. Furthermore, the lethality induced by strong chronic overexpression of *edin* using the UAS/Gal4 system implies that this gene can induce processes detrimental to a fly’s survival that could be affecting viability during persistent infections. Though Edin can be shown to cause pathology when overexpressed, it is difficult to produce clean evidence that this occurs during infection, because the overexpression of many genes can cause pathology; therefore it remains a suggestion.

**Is Edin an AMP?**

*Edin* shows several characteristics consistent with it being an AMP. First, it is strongly induced by infection; *edin* was the second most highly induced gene in wild-type flies following *L. monocytogenes* infection, and the most highly induced gene in *wntD* mutants. Second, *edin* is predicted to encode a short peptide and a processed form has been observed circulating in the hemolymph of infected flies [23]. However, *edin* also displays properties that would make it unique among AMPs, suggesting that it may be more broadly affecting physiology, perhaps in a cytokine-like role similar to that of *eiger*. For instance, the expression of this gene is required for normal survival following *L. monocytogenes* infection. While necessity for the signaling pathways controlling AMP expression is well documented, this is the first case of an individual putative AMP being necessary to fight infections [Ferrandon, 2007 #329]. This requirement during infection, combined with the toxicity observed upon overexpression suggests that Edin may be a powerful component of the immune response that must be tightly regulated to optimize survival. Further analysis of *edin* and other genes that are differentially regulated during pathogenesis could provide interesting clues into the complicated and evolving nature of the host-pathogen interaction.

**Materials and Methods**

**Drosophila strains**

The construction of *wntD* mutants was described previously [21]. Any reference to *wntD* mutant is the genotype *yw; wntD*Δ*ko*. References to ‘wild type’ refer to *yw; +/+; +/+ or *w*Δ*ko*; +/+; +/+ if so noted. *pUA* [UAS-*edin*] was constructed by amplifying the *edin* open reading frame using PCR, and cloning this fragment into the Xba-1 site of pPUAST [32]. UAS-RNAi(edin)2 was created at the open reading frame using PCR, and cloning this fragment into the element transformation techniques. Carrying expression constructs were created using standard p-element transformation techniques.

**Bacterial injections**

All injections were done using male flies aged one week post eclosion. A culture of *Listeria monocytogenes* was diluted to an OD(600) of 0.1, and a 25 nL volume was injected abdominally using a pulled glass needle as previously described [15]. Groups of 20 flies of each genotype were injected in an alternating manner to control for variability over time. Flies were maintained on non-yeasted, standard dextrose medium at 25°C, 65% relative humidity, and survival was monitored daily. *Micrococcus luteus* and *Salmonella typhimurium* was injected as described for *L. monocytogenes*. For experiments on the regulation of *edin*, flies of different genetic backgrounds were injected with a mixture of *M. luteus*, *L. monocytogenes*, and *E. coli*, each at an OD(600) of 0.1.

**Quantitative RT-PCR**

Groups of 6 flies were collected, crushed in 150 μL of Trizol reagent, and RNA was extracted according to the manufacturer’s recommendations. 1 μL RNA was used for subsequent reverse transcription using the ThermoScript RT-PCR system (Gibco BRL), following the manufacturer’s instructions and using a random hexamer as primer. Quantitative PCR was performed in a LightCycler (Roche), using the LightCycler FastStart DNA MasterPLUS SYBR green I kit (Roche) and following the manufacturer’s recommendations. Primers used for PCR were as follows:

- **edin**: TCCAGTGGCACCCTTTGTA and TAGTGTTCCGGATTGTGCGAA
- **eiger**: GATGGTCTGGATTCCATTGC and TAGTCTGGCACAACATCATC
- **ribosomal protein 15a**: TGGACCAAGAGGAGGCTAGG and GTTGGTGCATGGGTGGTA

**Gene expression profiling**

Groups of 30 *yw; wntD*Δ*ko* or *yw* flies (some previously infected with *Listeria monocytogenes* as described above) were collected in 1.5 mL microfuge tubes. Each experiment was done in triplicate, for 12 total samples. Conditions were: *yw* uninjected, *yw; wntD*Δ*ko* uninjected, *yw* 24 hours post *Listeria* infection, *yw; wntD*Δ*ko* 24 hours post *Listeria* infection. Flies were crushed in 1 mL Trizol reagent, and RNA was isolated using the manufacturer’s recommendations. 15 μg of each RNA sample was then used for cDNA synthesis, which was done using the one cycle cDNA synthesis (Affymetrix) and following the manufacturer’s recommendations. cDNA was also synthesized using the manufacturer’s protocol, and 20 μg was used for the subsequent fragmentation step. cRNA was hybridized to Affymetrix Drosophila Genome 2.0 arrays by the Stanford Protein and Nucleic Acid Biotechnology Facility (http://pan.stanford.edu). Arrays were analyzed using the Affymetrix GCOS software to produce normalized values for each probe set on each array.

**Clustering**

Clustering was performed on a dataset in which genes were included only if they were marked as “present” by GCOS in all 3 samples of at least one condition. Clustering was done using Cluster 3.0 for Mac OS X (http://bonsai.hims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm). Parameters used for clustering were: Data was log transformed and genes were centered. Data was filtered to include only genes where the difference between the highest and lowest values was greater than or equal to 1 (representing a two-fold change or greater). Hierarchical clustering was performed using the centroid linkage
algorithm. Clusters were viewed using Java Treeview software (http://genetics.stanford.edu/∼aickol/TreeView/). Gene identities and annotations shown in Tables S1 and S2 were retrieved using the NetAffx analysis webpage (http://www.affymetrix.com/analysis/index.affx).

Supporting Information

Table S1  Genes in cluster A
Found at: doi:10.1371/journal.ppat.1000111.s001  (0.11 MB DOC)

Table S2  Genes in cluster B
Found at: doi:10.1371/journal.ppat.1000111.s002  (0.21 MB DOC)

References

1. Khush RS, Leutler F, Lemaire B (2001) Drosophila immunity: two paths to NF-

2. Tanji T, Ip YT (2005) Regulators of the Toll and Imd pathways in the Drosophila

3. Brennan CA, Anderson KV (2004) Drosophila: the genetics of innate immune

4. De Gregorio E, Spellman PT, Trez P, Rubin GM, Lemaire B (2002) The Toll and

5. Irving P, Troder L, Heuer TS, Belvin M, Kopczynski C, et al. (2001) A genome-

6. Boutros M, Agaisse H, Perrimon N (2002) Sequential activation of signaling

7. Imler JL, Bulet P (2005) Antimicrobial peptides in Drosophila: structures,

8. Bettencourt R, Asha H, Dearolf C, Ip YT (2004) Hemolymph-dependent and -

9. Ligoxygakis P, Pelte N, Ji C, Leclerc V, Duvic B, et al. (2002) A serpin mutant

10. Qiu P, Pan PC, Govind S (1998) A role for the Drosophila Toll/Cactus pathway

11. Soderhall K, Cerenius L (1998) Role of the prophenoloxidase-activating system

12. Zettervall CJ, Anderl I, Williams MJ, Palmer R, Kurucz E, et al. (2004) A

13. Alarco AM, Marcil A, Chen J, Suter B, Thomas D, et al. (2004) Immune-

14. Brandt SM, Dionne MS, Khush RS, Pham LN, Vigdal TJ, et al. (2004) Secreted

15. Dionne MS, Ghori N, Schneider DS (2003) Drosophila melanogaster is a

16. Mansfield BE, Dionne MS, Schneider DS (2003) Exploration of host-pathogen

17. Beutler B, Rietschel ET (2003) Innate immune sensing and its roots: the story of

18. Beutler B, Milsark JW, Cerami AC (1985) Passive immunization against
cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin.
Science 228: 869–871.

19. Tracey KJ, Lowry SF, Beutler B, Cerami A, Albert JD, et al. (1986) Cachectin/
tumor necrosis factor mediates changes of skeletal muscle plasma membrane
potential. J Exp Med 164: 1368–1373.

20. Schneider DS, Ayres JS, Brandt SM, Costa A, Dionne MS, et al. (2007) Drosophila eiger mutants are sensitive to extracellular pathogens. PLoS Pathog

21. Gordon MD, Dionne MS, Schneider DS, Nusse R (2005) WntD is a feedback inhibitor of Dorsal/NF-kappaB in Drosophila development and immunity.
Nature 437: 746–749.

22. Ganguly A, Jiang J, Ip YT (2005) Drosophila WntD is a target and an inhibitor of the Dorsal/Twist/Sna1 network in the gastrulating embryo. Development.

23. Verleyen P, Baggerman G, D'Hertog W, Vierstraete E, Husson SJ, et al. (2006) Identification of new immune induced molecules in the haemolymph of Drosophila melanogaster by 2D-nanoLC MS/MS. J Insect Physiol 52: 379–383.

24. De Gregorio E, Spellman PT, Rubin GM, Lemaitre B (2001) Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc Natl Acad Sci U S A 98: 12590–12595.

25. Eisten MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95: 14963–14968.

26. Ayres JS, Freitag N, Schneider DS (2008) Identification of Drosophila mutants altering defense of and endurance to Listeria monocytogenes infection. Genetics 178: 1807–1815.

27. Zambon RA, Nandakumar M, Vakharia VN, Wu LP (2005) The Toll pathway is important for an antiviral response in Drosophila. Proc Natl Acad Sci U S A 102: 1757–1762.

28. Bischoff V, Vignal C, Duvic B, Boneca IG, Hoffmann JA, et al. (2006) Downregulation of the Drosophila Immune Response by Peptidoglycan-

29. Choe KM, Werner T, Steven S, Hultmark D, Anderson KV (2002) Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila. Science 296: 359–362.

30. Gottar M, Gobert V, Michel T, Belvin M, Duyck G, et al. (2002) The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature 416: 648–644.

31. Kaneko T, Golenbock D, Silverman N (2000) Peptidoglycan recognition by the Drosophila Immd pathway. J Endotoxin Res 11: 383–389.

32. Brand AH, Manoukian AS, Perrimon N (1994) Ectopic expression in Drosophila. Methods Cell Biol 44: 635–654.

33. Lee YS, Carthew RW (2005) Making a better RNAi vector for Drosophila: use of intron spaces. Methods 39: 322–329.