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The Drosophila Protein Mustard Tailors the Innate Immune Response Activated by the Immune Deficiency Pathway

Zhipeng Wang, Cristin D. Berkey, and Paula I. Watnick

In this study, we describe a Drosophila melanogaster transposon insertion mutant with tolerance to Vibrio cholerae infection and markedly decreased transcription of dipterinc as well as other genes regulated by the immune deficiency innate immunity signaling pathway. We present genetic evidence that this insertion affects a locus previously implicated in pupal eclosion. This genetic locus, which we have named mustard (mtd), contains a LysM domain, often involved in carbohydrate recognition, and a TLDc domain of unknown function. More than 20 Mtd isoforms containing one or both of these conserved domains are predicted. We establish that the mutant phenotype represents a gain of function and can be replicated by increased expression of a short, nuclearly localized Mtd isoform comprised almost entirely of the TLDc domain. We show that this Mtd isoform does not block Relish cleavage or translocation into the nucleus. Lastly, we present evidence suggesting that the eclosion defect previously attributed to the Mtd locus may be the result of the unopposed action of the NF-kB homolog, Relish. Mtd homologs have been implicated in resistance to oxidative stress. However, to our knowledge this is the first evidence that Mtd or its homologs alter the output of an innate immunity signaling cascade from within the nucleus. The Journal of Immunology, 2012, 188: 3993–4000.

The epithelia of most animals are continually exposed to microorganisms. By necessity, these animals have evolved signaling pathways that enable symbiotic interactions with beneficial microbes and activate an innate immune defense against invading pathogens.

The common fruit fly Drosophila melanogaster harbors two distinct innate immune signaling pathways, namely the immune deficiency (Imd) pathway, which responds primarily to Gram-negative bacteria, and the Toll pathway, which responds primarily to Gram-positive bacteria and fungi (1, 2). Both of these pathways regulate nuclear translocation of NF-kB homologs, which activate the transcription of many genes, including those that encode small cationic antimicrobial peptides (AMPs).

Three NF-kB homologs are encoded in the Drosophila genome. Two of these, Dorsal and Dorsal-related immunity factor, respond to signaling through the Toll pathway (3). The third NF-kB homolog, Relish, comprised of an N-terminal Rel homology domain (RHD) and a C-terminal ankyrin repeat domain, responds to signaling through the IMD pathway, which is initiated by receptor protein recognition of meso-diaminopimelic acid-type peptidoglycan. Through a series of intermediates, this leads to activation of the caspase 8 homolog Dredd and the IkK complex consisting of the catalytic subunit IkKB, encoded by ird5, and the regulatory subunit IkKγ, encoded by Kenny (key). Phosphorylation of Relish by the IkK complex at residues 528 and 529 is required for activation of Relish targets. Dredd is required for cleavage of the Relish C-terminal inhibitory ankyrin repeat domain, allowing nuclear translocation of a 68 kDa N-terminal RHD-containing fragment and activation of an immune response against Gram-negative and some Gram-positive bacterial pathogens (4–7).

Many studies have demonstrated that, in addition to generation of reactive oxygen species by the host (8, 9), IMD pathway signaling plays a role in the Drosophila innate immune response to both commensal and pathogenic intestinal bacteria (10–12). To protect the host against its own immune response, signaling through this pathway is tightly regulated (12–18).

Vibrio cholerae, a Gram-negative, halophilic bacterium, is responsible for the severe diarrheal disease cholera (19). Furthermore, it has been associated with both marine and terrestrial arthropods in the environment (20–24), and arthropods have been proposed as both reservoirs and vectors of V. cholerae. We developed D. melanogaster as a model in which to study the interaction of V. cholerae with the arthropod intestine (25) and previously reported that IMD pathway mutants have increased tolerance to oral V. cholerae infection (26).

As part of a genetic screen for host susceptibility factors, we identified a mutant with increased tolerance to oral V. cholerae infection and a transcriptional profile similar to that of an IMD pathway mutant. This mutant, which we have named Mustard (Mtd), carries a P-element insertion in a complex genetic locus encoding conserved LysM and TLDc domains. LysM domains are often involved in carbohydrate recognition (27). Although the TLDc domain has been associated with resistance to oxidative stress, its mechanism of action is unknown (28). The Mtd locus is predicted to give rise to 21 transcripts containing one or both of these conserved domains (29). We show that the phenotype of the Mtd mutant represents a gain of function and can be reproduced.
by expression of a naturally localized isoform containing only the TLDC domain. TLDC domains are widespread in eukaryotes.

In this study, to our knowledge we present the first evidence for modulation of the IMD innate immune signaling pathway by a TLDC domain-containing protein.

Materials and Methods

Bacterial strains, fly stocks, and growth media

MO10, a V. cholerae O139 clinical isolate, was used in all Drosophila infections (30). Either w1118 or yw stocks obtained from the Bloomington Drosophila stock center at Indiana University were used as controls. The details of other fly lines used in these experiments are available upon request. Other than the decreased rates of eclosion observed for insertion mutants with global defects in Mtd transcription, no developmental defects were noted for the fly lines used in these experiments. D. melanogaster strains were reared at 24˚C on standard Drosophila medium, and bacterial strains were propagated in Luria–Bertani (LB) broth supplemented with streptomycin (100 μg/ml) at 27˚C.

Transposon localization

Inverse PCR was used to identify the P-element insertion site of the mtdΔVisD mutant as follows. Genomic DNA was prepared from 25 adult flies using a QIAamp DNA Mini Kit (Qiagen) and then digested with MluI. Resulting PCR fragments were self-ligated overnight at 16˚C using T4 DNA ligase (Invitrogen) to create a mixture of circular fragments. DNA flanking the 5’ end of the transposon was amplified from this mixture using the outward facing primer pair Pwsh1 (GTAAACGCTAT-CACTCCGAAACGGCTACA) and Pcla1 (CCACCAAGGCTGGCTGCC-CAACA). DNA flanking the 3’ end of the transposon was amplified using the primer pair Psh1 (CAATCAATCTCCGTCCTCACTCA) and Pyl1 (CCTTACAGCTGGCCTGGTTGAAT). PCR products were sequenced and aligned with the Drosophila genome sequence available from the Berkeley Drosophila Genome Project.

Transgenic fly construction

The mtd-RC transcript was reverse-transcribed from Drosophila RNA prepared as described below using a primer specific to the 3’ end of the RC transcript and the SuperScript III First-Strand synthesis system (Invitrogen). The resulting cDNA was amplified with a 5’ primer including an EcoRI site and a reverse primer including an XhoI site. This PCR product was inserted into pCR2.1-TOPo using a TOPO TA cloning kit (Invitrogen) and then excised and ligated into pUAST using the EcoRI and XhoI restriction sites. A pUAST vector encoding hemagglutinin (HA)-tagged Mtd-RC was created similarly except that an HA tag was added in two steps by PCR amplification with primers encoding the tag. We first confirmed the presence of the plasmid-RC transcript by reverse-transcribing and sequencing this transcript. Then, mtd-RC and mtd-RC-HA transcripts were amplified from the pUAST plasmids containing either mtd-RC or mtd-RC-HA, respectively, using a forward primer including a 5’ EcoRI site and a reverse primer including a 3’ XhoI site. After digestion with EcoRI and XhoI, these products were ligated into pUAST. All inserts were confirmed by sequence analysis. Plasmids were sent to Rainbow Transgenics for injection into w1118 embryos. Homozygous transgenic fly stocks were generated from these offspring.

Oral and systemic bacterial infections

Flies were infected orally as previously described (25). Briefly, three fly vials were prepared with a cellulose acetate plug containing 2 ml of an overnight culture of V. cholerae diluted in a 1:10 ratio in LB broth. Thirty male flies were divided equally between these three vials. Numbers of visible flies in each vial were recorded at least once each day. Survival curves were constructed, and log-rank analysis was used to determine statistical significance. Reproducibility of all survival data was confirmed in at least two independent experiments.

Septic injury was used to produce systemic infection for experiments as noted. In this model, 30 flies were anesthetized with CO2 and pricked in the dorsal thorax with a fine needle (Fine Science Tools) dipped in a solution containing either fresh LB broth or LB broth harboring an overnight culture of V. cholerae. After septic injury, flies were returned to food vials, where they remained until the specified time.

Measurements of bacterial load

Quantification of bacterial load within infected flies was assayed as previously described (26). After 24 h of V. cholerae ingestion, 10 flies were collected and homogenized in 200 μl PBS. Dilutions of the suspension were plated on LB agar supplemented with streptomycin. CFUs per fly were enumerated. No colonies were cultured from flies fed LB broth alone, suggesting that all bacteria retrieved from LB agar plates supplemented with streptomycin were V. cholerae. Statistical significance was calculated using a Mann–Whitney U test.

Quantitative RT-PCR

Flies were homogenized in TRIzol reagent (Invitrogen). Total RNA was extracted once, treated with DNase I, and then extracted a second time. Purified RNA (1 μg) was used as a template for synthesis of cDNA using a QuantiTect reverse transcription system (Qiagen). The resulting cDNA was used for quantitative PCR with iTaq SYBR Green supermix with carboxy-X-rhodamine (ROX; Bio-Rad) and 2 pmol relevant primers in a 20-μl reaction volume. The sequence of primers used for quantitative RT-PCR (qRT-PCR) is available on request. The experiments were conducted with a StepOnePlus PCR system (Applied Biosystems). The transcript level of each gene was quantified by comparison with a standard curve and normalized to the reference gene rp49. Ten to 15 flies were used per biological replicate, and three biological replicates were performed for each experiment. Error bars represent the SEM, and statistical significance was calculated using a Student t test. Each experiment was performed at least twice with the exception of those in Supplemental Fig. 3, which were performed once.

In control yw flies, we found that, after 24 h of V. cholerae ingestion, dipt expression increased ~35-fold and remained at this level over several days. Therefore, in oral infections, dipt transcription was measured after 24 h. Six hours after septic injury, we found that dipt transcription had increased ~230-fold. It continued to increase over the next 3 h and then was maintained at 300 times baseline (data not shown). Therefore, for septic injury, dipt expression was measured after 6 h.

Western analysis

Protein extracts for Western analysis were prepared as follows. Ten adult flies were homogenized in a 200 μl volume of NP-40 cell lysis buffer (Invitrogen) supplemented with 1 mM PMSF and a protease inhibitor tablet (Roche). Extracts were spun at 14,000 rpm for 10 min at 4˚C to pellet debris. Sixty microliters of the resulting supernatant was then combined with 15 μl Lane Marker Reducing Sample Buffer (Pierce), boiled for 5 min, and spun at 14,000 rpm for 1 min. Proteins in this mixture were separated on an 8% Precise protein gel (Invitrogen). Full-length Relish and the products of its proteolysis were visualized with the following two primary Abs: 1) a mouse IgG mAb to the C terminus of Relish (Developmental Studies Hybridoma Bank), and 2) a polyclonal rabbit IgG Ab raised against a peptide fragment within the N-terminal RHD domain (Pacific Immunology). Each primary Ab was used in a 1:1000 dilution. Anti-mouse or anti-rabbit IgG secondary Ab conjugated to horseradish peroxidase (Amersham) was used as appropriate in a 1:15,000 dilution. The HRP-conjugated secondary Abs were visualized by chemiluminescence with the ECL Plus Western blotting system (Amersham Biosciences).

Immunofluorescence

Fly tissues were dissected in PBS, transferred immediately to 4% formaldehyde in PBS, and incubated for 30 min at room temperature. Tissue was then rinsed three times for 10 min each in PBS supplemented with 0.1% Triton X-100 and 1% BSA and incubated overnight with one of the following primary Abs: mouse anti-HA (1:100; Santa Cruz Biotechnology), mouse anti-Flag (1:500; Sigma-Aldrich), or rabbit anti-HA (1:100; Santa Cruz Biotechnology). After rinsing, a second overnight incubation was performed with one of the following secondary Abs: Alexa 488 goat anti-mouse IgG (1:100; Invitrogen), Dylight 549 goat anti-mouse IgG (1:100; Jackson ImmunoResearch Laboratories), or Alexa 488 goat anti-rabbit IgG (1:200; Invitrogen). The tissues were rinsed again with addition of DAPI (1 μg/ml) to the last wash. Tissues were mounted in Vectashield and examined using an LSM700 confocal microscope.

Microarray analysis

RNA was prepared as described above except that 100 flies were used. This RNA was supplied to the Molecular Genetics Core Facility at Children’s Hospital Boston where it was labeled and hybridized to a GeneChip Drosophila Genome 2.0 Array (Affymetrix) according to the manufacturer’s instructions. Data analysis was carried out using GeneSpring software (Silicon Genetics, Redwood City, CA). Statistical significance was determined using a one-way ANOVA with a threshold p value of 0.05. Microarray data have been deposited in the Gene Expression Omnibus database (accession no. GSE35439; http://www.ncbi.nlm.nih.gov/geo/).
Results

Identification of a transposon-insertion mutant with increased tolerance to oral V. cholerae infection

In a forward genetic screen of homozygous viable Drosophila transposon-insertion mutants, we identified a line, P[EPgy2] (3); 82FdEY04695, that, similar to IMD pathway mutants, showed prolonged survival in the face of oral V. cholerae infection (Fig. 1A) (26). We named this mutant Mustard. The bacterial load of the mtdEY04695 mutant was similar to that of IMD pathway mutants and slightly less than that of control flies (Fig. 1B). Because the mtdEY04695 mutant was comparably susceptible to colonization by V. cholerae but less susceptible to lethal infection than were control flies, we describe it as tolerant to V. cholerae infection (31).

Although Relish cleavage proceeds normally, diptericin transcription is decreased in the mtdEY04695 mutant

We questioned whether IMD pathway signaling might be blocked in the mtdEY04695 mutant. The IMD pathway directly regulates expression of the antimicrobial peptide Diptericin (32). Therefore, to evaluate signaling through the IMD pathway, we compared dipt transcription in the mtdEY04695 mutant to that in control flies in the presence and absence of oral or systemic infection with V. cholerae. As shown in Fig. 2, dipt levels were dramatically reduced in uninfected, orally infected, and systemically infected mtdEY04695 mutant flies.

Because dipt transcription was decreased in the mtdEY04695 mutant, we hypothesized that IMD pathway signaling might be blocked prior to Relish cleavage. To examine this possibility, we performed Western analysis on protein extracts derived from control flies and mtdEY04695 mutant flies before and after systemic infection with V. cholerae. As shown in Fig. 3, Relish and its cleavage products were equally abundant in extracts from wild-type and mtdEY04695 mutant flies postinfection. As a control, neither full length Relish nor its cleavage products were present in a relE20 mutant. This suggests that, in the mtdEY04695 mutant, signaling through the IMD pathway proceeds past the point of Relish cleavage.

In multiple experiments, Rel68 but not Rel49 appeared to be increased in the uninfected mtdEY04695 mutant as compared with control flies. This may suggest that the Rel68 peptide is less susceptible to proteolytic degradation in the mtdEY04695 mutant.

Similarities and differences in gene regulation in an IMD pathway mutant and the mtdEY04695 mutant

Because cleavage of Relish proceeds normally in the mtdEY04695 mutant, we questioned whether transcription of other AMPs was repressed in this mutant. Therefore, we measured the transcript levels of a number of AMPs in flies orally infected with V. cholerae (Fig. 4). Interestingly, although attacin, cecropin, defensin, drosocin, and metchnikowin transcripts were decreased in the IMD pathway mutant, levels of these transcripts in the mtdEY04695 mutant were similar to those in control flies. Transcription of drosomycin, which is regulated by the Toll pathway, was slightly decreased but significantly reduced in both mutants.

To determine whether there might be other genes whose transcription was similarly altered in an IMD pathway mutant and the mtdEY04695 mutant, we compared the transcriptomes of orally infected key1 and mtdEY04695 mutant flies with those of w1118.
control flies by microarray analysis. Three hundred ninety-eight genes were differentially regulated in the mtd\textsuperscript{EY04695} mutant and 434 genes were differentially regulated in the key\textsuperscript{1} mutant as compared with control flies. Of these, 40% were similarly regulated in mtd\textsuperscript{EY04695} and key\textsuperscript{1} mutant flies (Supplemental Table 1). For a small group of the most highly regulated genes, we confirmed the microarray results by qRT-PCR. As shown in Fig. 5, transcript levels of Edin, CG12498, and CG10725 were similarly regulated in the two mutants, whereas levels of CG13659 were increased.

The effect of the transposon insertion on the mtd locus is complex

The mtd\textsuperscript{EY04695} mutant harbors a P-element insertion within the mtd locus, which is predicted to encode 20 unique isoforms as a result of alternative splice sites and alternative promoters (29). Mtd isoforms include one or both of the conserved LysM and TLDc domains encoded within the Mtd locus (Fig. 6A). The LysM domain is present in prokaryotic and eukaryotic proteins that bind carbohydrates such as peptidoglycan and chitin (27, 33–35), whereas the TLDc domain has no known function but has been associated with resistance to oxidative stress (28, 36–41). Eleven of the predicted mtd transcripts include both LysM and TLDc domains (LysM/TLDc), nine of the predicted shorter transcripts include only the TLDc domain (TLDc-only), and one predicted transcript, RD, includes only the LysM domain (LysM-only).

Transposon mapping by inverse PCR and subsequent sequence analysis indicated that the P-element was located 200 bp upstream of the start codon of a transcript encoding the TLDc-only isoform, mtd-RC (Fig. 6A).

Given the complexity of the mtd locus, our first goal was to identify mtd transcripts whose abundance was altered in the mtd\textsuperscript{EY04695} mutant. We hypothesized that the insertion would decrease levels of mtd-RC and might also alter the abundance of other mtd transcripts. To test this hypothesis, we identified amplicons (shown in Fig. 6A) that allowed us to uniquely measure the abundance of the TLDc-only transcripts, mtd-RC and mtd-RH, and the LysM-only transcript mtd-RD. Additionally, we measured the overall abundance of LysM/TLDc transcripts using primers just downstream of the LysM domain. We compared transcript levels in control flies and the mtd\textsuperscript{EY04695} mutant. Whereas transcription of mtd-RC in the mutant was 12-fold less than that in control flies, levels of mtd-RH and all long transcripts were increased by 66.8 and 37.9%, respectively (Fig. 6B, 6C, 6E). Mtd-
RD transcription was unchanged in the mtd<sup>EY04695</sup> mutant (Fig. 6D). Based on these data, we concluded that the mtd<sup>EY04695</sup> mutant phenotype could result either from decreased levels of mtd-RC or from increased levels of mtd-RH and the longer LysM/TLDe transcripts.

Because the mtd<sup>EY04695</sup> mutant was more tolerant to oral infection with <i>V. cholerae</i>, we were particularly interested in mtd transcripts that were expressed in the intestine. We found high levels of mtd-RH, mtd-RC, and full-length transcripts in the intestine. In contrast, expression of mtd-RD in the intestine was negligible (Supplemental Fig. 1). Furthermore, oral infection did not alter the levels of any of the measured transcripts either in whole flies or specifically in the intestine (Supplemental Fig. 1).

**Precise excision suggests that the transposon insertion is responsible for the mtd<sup>EY04695</sup> mutant phenotype**

We first used sequence analysis to establish that the extremely low expression of dipt in the mtd<sup>EY04695</sup> mutant was not the result of a mutation in the promoter or coding sequence of the dipt gene (data not shown). We then confirmed that the phenotype of the mtd<sup>EY04695</sup> mutant was, in fact, due to the P-element insertion by crossing the mtd<sup>EY04695</sup> mutant with flies carrying the P-element transposase. PCR amplification of the insertion site was used to screen for precise excision lines. This was confirmed by sequence analysis. Precise excision lines were then backcrossed to the parental strain to generate flies with a comparable genetic background. Two independent precise excision lines (EP13 and EP18) were studied in detail.

Transcription of both mtd-RC and mtd-RH in the EP13 and EP18 precise excision lines was significantly different from that of the mtd<sup>EY04695</sup> mutant and closer to that of control flies (Supplemental Fig. 2A, 2B). The dipt transcription in precise excision lines was also closer to that of control flies (Supplemental Fig. 2C). We compared the susceptibility of precise excision lines to oral <i>V. cholerae</i> infection with that of control flies. The susceptibility to infection of both the EP13 and EP18 precise excision lines was significantly greater than that of the mtd<sup>EY04695</sup> mutant. However, only the susceptibility of the EP18 line approached that of control flies (Supplemental Fig. 2D, 2E). Taken together, these results strongly suggest that the P-element insertion in the mtd locus is responsible for the phenotype of the mtd<sup>EY04695</sup> mutant.

**The mtd<sup>EY04695</sup> mutant phenotype is not due to a decrease in the mtd-RC transcript**

We hypothesized that the mtd<sup>EY04695</sup> mutant phenotype could be due either to a decrease in transcription of mtd-RC or to increased levels of another transcript. To determine whether decreased mtd levels could be responsible for the mtd<sup>EY04695</sup> mutant phenotype, we generated a hemizygous mutant by crossing the mtd<sup>EY04695</sup> fly line with a deficiency strain lacking the mtd gene region (Df(3R)3-

**Figure 7.** Overexpression of mtd-RH but not mtd-RC blocks activation of dipt transcription. (A) qRT-PCR measurement of mtd-RC transcript levels in a strain with ubiquitous overexpression of mtd-RC (UAS-Mtd-RC/Da-Gal4) and a driver-only control (+/Da-Gal4). (B) qRT-PCR measurement of dipt transcript levels in a strain with ubiquitous overexpression of mtd-RC (UAS-Mtd-RC/Da-Gal4) and a driver-only control (+/Da-Gal4). (C) Same experiment as (B) performed with flies infected with <i>V. cholerae</i> by septic injury. (D) qRT-PCR measurement of mtd-RH transcript levels in a strain with ubiquitous overexpression of mtd-RH (UAS-Mtd-RH/Da-Gal4) and a driver-only control (+/Da-Gal4). (E) Same experiment as (D) performed with flies infected with <i>V. cholerae</i> by septic injury.

**Figure 8.** Overexpression of mtd-RH but not mtd-RC increases tolerance to oral <i>V. cholerae</i> infection. Fractional survival of flies with (A) ubiquitous expression of mtd-RC (UAS-Mtd-RC/Da-Gal4) or mtd-RH (UAS-Mtd-RH/Da-Gal4) compared with driver-only control flies (+/Da-Gal4) and (B) midgut-only expression of mtd-RC (UAS-Mtd-RC/NP1-Gal4) or mtd-RH (UAS-Mtd-RH/NP1-Gal4) compared with driver-only control flies (+/NP1-Gal4). Log-rank analysis demonstrated a statistically significant difference between survival of control flies and flies expressing mtd-RH either ubiquitously (<i>p < 0.0001</i>) or in the midgut alone (<i>p < 0.0001</i>) but not between survival of control flies and flies expressing mtd-RC ubiquitously (<i>p > 0.05</i>) or in the midgut alone (<i>p > 0.05</i>).


4, Df). As expected, transcription of mtd-RC in the resulting hemizygous fly (mtd\textsuperscript{EY04695}/Df) was decreased (Supplemental Fig. 3A). However, dipt transcription in this hemizygote was similar to that of the heterozygote control (Supplemental Fig. 3B). Furthermore, susceptibility to V. cholerae infection was comparable to that of control flies (Supplemental Fig. 3C). This suggested to us that a decrease in the level of the mtd-RC transcript was not the basis of the mtd\textsuperscript{EY04695} mutant phenotype.

To further support this conclusion, we examined a series of mutants from the Harvard Exelixis stock collection carrying insertions in the mtd locus (Fig. 6A). In these mutants, levels of the mtd-RC and LysM/TLDc transcripts were reduced, whereas mtd-RH transcription was not altered (Supplemental Fig. 3D–F). In contrast to the mtd\textsuperscript{EY04695} mutant, these mutants were all more susceptible to infection (Supplemental Fig. 3G). Taken together, these results suggest that the mtd\textsuperscript{EY04695} phenotype is not the result of a decrease in mtd-RC transcription.

**Overexpression of mtd-RH but not mtd-RC phenocopies the mtd\textsuperscript{EY04695} mutant**

Next, we examined the possibility that the mtd\textsuperscript{EY04695} phenotype might result from an increase in the level of mtd-RH. We confirmed the presence of RC and RH transcripts and generated transgenic flies encoding a wild-type mtd-RH allele downstream of a UAS element controlled by Gal4. As a control, we generated transgenic flies carrying a similar mtd-RC construct. Mtd isoform C (Mtd-PC) differs from isoform H (Mtd-PH) only by the presence of 70 additional amino acids at its N terminus.

When induced by the ubiquitous driver Daughterless (Da-Gal4), transcription of mtd-RC and mtd-RH increased >10-fold in comparison with the Gal4 control (Fig. 7A, 7D). Overexpression of mtd-RC did not alter dipt transcription either in uninfected or systemically infected flies (Fig. 7B, 7C). In contrast, overexpression of mtd-RH significantly repressed dipt transcription in uninfected and systemically infected flies (Figs. 7E, 7F).

We also tested the susceptibility of these flies to V. cholerae infection. Overexpression of mtd-RH but not mtd-RC both ubiquitously and in the midgut prolonged survival (Fig. 8). These results support the conclusion that the phenotype of the mtd\textsuperscript{EY04695} mutant is due, at least in part, to an increase in the level of the mtd-RH transcript.

**Mtd-PC and Mtd-PH exhibit distinct subcellular distributions**

Mtd-PC and Mtd-PH differ only in 70 aas at their N termini; however, overexpression of these two proteins leads to very different effects on IMD pathway signaling and resistance to oral infection. Because Rel68 relocates to the nucleus, we hypothesized that the different functions of these two transcripts could be the result of distinct subcellular distributions. To examine this hy-
ypothesis, we generated flies carrying wild-type mtd-RC or mtd-RH alleles with a C-terminal human influenza HA tag added down-stream of a Gal4-UAS promoter. We used the Da-Gal4 driver to express these proteins in the midgut. Interestingly, although Da-Gal4 is considered a ubiquitous driver, the transgenes were not equally expressed in all midgut epithelial cells, leading to a mosaic pattern (Fig. 9). A similar pattern was noted when Da-Gal4 was used to drive a UAS-GFP transgene, demonstrating that this mosaic pattern of expression is not specific to Mtd (data not shown).

Mtd-PC and Mtd-PH were visualized by immunofluorescence both in the larval fat body and in the adult midgut. Whereas isoform C was observed exclusively in the cytoplasm of larval fat body and adult midgut epithelial cells (Fig. 9A, 9C), isoform H was also present in the nucleus (Fig. 9B, 9D). Thus, despite the high similarity of Mtd-PC and Mtd-PH, these proteins have distinct distributions within the cell. This is likely the basis for their distinct effects on targets of the IMD pathway.

mtd-RH rescues the eclosion defect that results from rel68 overexpression

To determine whether Mtd-PC or Mtd-PH altered the subcellular distribution of Rel68, we coexpressed tagged alleles of mtd-RH and rel68 and visualized the subcellular locations of these two proteins in larval fat body cells by immunofluorescence. As shown in Fig. 10A–C, neither Mtd-PC nor Mtd-PH blocked nuclear localization of Rel68. However, while rearing Rel68 overexpression flies, we noticed that although these flies developed normally, many of them were unable to eclose (Figure 10D, 10E). Interestingly, co-overexpression of mtd-RH and rel68 rescued this eclosion defect. Coexpression of mtd-RC with rel68 did not mitigate the eclosion defect and, in fact, appeared to exacerbate it. These data support a genetic interaction of Mtd-PH but not Mtd-PC with the NF-κB homolog Relish.

Discussion

In a screen for factors that alter Drosophila susceptibility to oral V. cholerae infection, we identified an infection-tolerant transposon-insertion mutant in the complex l(3)82Fd locus, which we have renamed mustard (mtd). Subsequent experiments suggested that the phenotype of this mutant was the result of increased expression of a short Mtd transcript containing only a TLDc domain. The mtd locus was previously described as an edysione-activated, late puff gene whose mutation led to the inability of adult flies to eclose (42, 43). It encodes at least 20 unique isoforms, including a LysM-only isoform, several TLDc-only isoforms, and several with both domains. In this study, we report a novel function for a short, nuclearly localized Mtd isoform in tailoring of the IMD pathway-activated innate immune response.

The Mtd homolog Oxr1 has been shown to protect cells against reactive oxygen species (ROS), which are produced during intestinal infection as well as in neurodegenerative disease, by increasing the transcription of genes that scavenger ROS such as catalase and glutathione peroxidase (9, 41, 44). Furthermore, investigators have shown that C167S of the mouse Mtd homolog itself can be oxidized by H2O2 (41). This residue (C167) is conserved in Mtd-PH. We previously showed that breakdown of the Drosophila midgut epithelium occurs during V. cholerae infection of wild-type Drosophila and presented evidence that increased cell turnover within the intestines of IMD pathway mutants might enhance tolerance to infection (26). Therefore, we initially hypothesized that breakdown of the fly intestinal epithelium during V. cholerae infection was the result of ROS production by dual oxidase and that Mtd increased host tolerance by protecting the intestinal epithelium against oxidative stress. However, RNA interference knockdown of the dual oxidase transcript, over-expression of the immune regulated catalase, and oral administration of glutathione did not prolong survival of orally infected control flies (data not shown), suggesting that ROS itself is not an important factor in fly mortality and that Mtd does not increase host tolerance by directly or indirectly scavenging ROS generated by the host immune response. However, it is possible that oxidation of Mtd by host-derived ROS could modulate its function.

We also considered that the low level of diptericin transcription in the mtd mutant reflected a less invasive infection leading to decreased activation of the IMD pathway. However, we observed that, in oral infection, the abundance of other IMD-regulated AMP transcripts was similar to that of control flies, suggesting that the IMD pathway is similarly activated in control and mtd<sup>Ev04695</sup> mutant flies. Alternative mechanisms for the observed effect of Mtd-PH on transcription of genes in the Relish regulon include 1) interaction with Rel68 at specific promoters, 2) interaction with DNA at a subset of Rel68-regulated promoters, 3) interaction with another protein that modulates transcription of genes in the Relish regulon, or 4) direct modulation of transcription of a protein that affects Relish function by binding to DNA. These hypotheses are currently under investigation.

The loci encoding the two human homologs of Mtd, Oxr1 and NCOA7, are also predicted to give rise to multiple transcripts and isoforms (28, 38, 40, 45). Interestingly, although there is considerable variability in the central regions of the fly and human homologs, the LysM and TLDc domains of these proteins are >50% identical. Furthermore, the TLDc domains of these proteins are much more similar to each other than they are to other TLDc domain-containing proteins in their respective genomes, suggesting that they may function similarly in flies and humans. Therefore, this work provides a rationale for the systematic investigation of the short isoforms of Oxr1 and NCOA7. Such studies may yield additional nuclearly localized isoforms that have new and important functions in regulation of gene transcription and modulation of the innate immune response in mammals.

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Disclosures

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