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Mutations in the IMD Pathway and Mustard Counter *Vibrio cholerae* Suppression of Intestinal Stem Cell Division in *Drosophila*

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**ABSTRACT** *Vibrio cholerae* is an estuarine bacterium and an intestinal pathogen of humans that causes severe epidemic diarrhea. In the absence of adequate mammalian models in which to study the interaction of *V. cholerae* with the host intestinal innate immune system, we have implemented *Drosophila melanogaster* as a surrogate host. We previously showed that immune deficiency pathway loss-of-function and mustard gain-of-function mutants are less susceptible to *V. cholerae* infection. We find that although the overall burden of intestinal bacteria is not significantly different from that of control flies, intestinal stem cell (ISC) division is increased in these mutants. This led us to examine the effect of *V. cholerae* on ISC division. We report that *V. cholerae* infection and cholera toxin decrease ISC division. Because IMD pathway and Mustard mutants, which are resistant to *V. cholerae*, maintain higher levels of ISC division during *V. cholerae* infection, we hypothesize that suppression of ISC division is a virulence strategy of *V. cholerae* and that accelerated epithelial regeneration protects the host against *V. cholerae*. Extension of these findings to mammals awaits the development of an adequate experimental model.

**IMPORTANCE** Here we show that *Vibrio cholerae* and cholera toxin suppress intestinal stem cell (ISC) division. This is the first evidence of manipulation of ISC division by *V. cholerae* and demonstrates the utility of the *Drosophila* model in generating novel hypotheses regarding the interaction of *V. cholerae* with the intestinal epithelium. Furthermore, we add to the body of data suggesting that the IMD pathway and the Mustard protein modulate ISC division independently of the overall load of commensal intestinal bacteria.

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*Vibrio cholerae* is a natural inhabitant of estuarine environments and a noninvasive intestinal pathogen that causes severe secretory diarrhea through the action of cholera toxin (CTX). The available mammalian models of cholera are limited because humans are the only known nonneonatal mammals that become colonized with *V. cholerae* in the absence of chemical or surgical intervention (1). To uncover new capabilities of this pathogen, we have developed *Drosophila melanogaster* as a model in which to study the interaction of *V. cholerae* with the intestinal innate immune system (2–5).

The *Drosophila* midgut, which functions in nutrient absorption in a manner analogous to that of the mammalian small intestine, consists of a single layer of enterocytes that is regenerated from intestinal stem cells (ISCs) that populate the base of the epithelium (6, 7). The intestinal epithelium is separated from the lumen by the peritrophic membrane, a structure composed of polysaccharides and proteins, which lines this part of the intestine. This membrane is the equivalent of the intestinal mucous that lines the mammalian intestine.

Much has been learned about the interplay among the physical, chemical, and cellular branches of the intestinal innate immune system in the invertebrate model *D. melanogaster* (8). For instance, the peritrophic membrane presents a physical barrier to bacterial penetration (9). Dual oxidase contributes to a chemical barrier through the generation of toxic reactive oxygen species (ROS) in the gut (10), while intestinal catalase protects the enterocytes themselves against damage by ROS (11). Last, enterocytes secrete antimicrobial peptides when the immune deficiency (IMD) signaling pathway is activated (12).

Signaling through the IMD pathway is initiated by the binding of bacterial peptidoglycan to a cell-associated receptor (13). Adaptors are then recruited, leading to phosphorylation of the IKK complex and activation of the caspase 8 homolog Dredd. The IKK complex, which consists of IRD5, a catalytic subunit, and Kenny (Key), a regulatory subunit, phosphorylates the NF-κB homolog Relish. Dredd is required for cleavage of Relish and may be the protease responsible for this process (14, 15). Translocation of Relish into the nucleus leads to activation of gene transcription. While neither cleavage nor phosphorylation is required for nuclear translocation of Relish, these modifications likely modulate the spectrum of Relish-regulated genes (16, 17).

*V. cholerae* infection of the arthropod intestine is distinct from other bacterial infections of the fly that have been studied. In contrast to its protective role in other bacterial infections, the IMD
pathway increases susceptibility to *V. cholerae* infection (3, 12). A similar phenotype is observed in a mutant with increased expression of the protein Mustard (mtd^EY0495; mtd^GOF), which regulates the transcription of a subset of IMD pathway target genes (2).

The mtd locus is essential for eclosion (18). It contains a LysM domain predicted to be involved in carbohydrate recognition and a TLDc domain whose function is unknown. Mtd has two close mammalian homologs, Oxr1 and NCOA7, that have been implicated in resistance to oxidative stress (19–25). Like Oxr1 and NCOA7, the Mtd locus encodes multiple unique isoforms, including 11 that contain both LysM and TLDc domains, one that contains only the LysM domain, and 10 that contain only the TLDc domain. We previously showed that overexpression of the TLDc-only isoform, Mtd-RH, is sufficient to repress the transcription of genes coregulated by Mtd and the IMD pathway (2). However, the mechanism of resistance of the mtd^GOF mutant to *V. cholerae* infection was not elucidated.

The goal of this work was to further investigate the mechanism by which mtd^GOF and IMD pathway mutants are protected against *V. cholerae* infection. Here we show that ISC division is increased in both of these mutants. In contrast, *V. cholerae* and its principal virulence factor CTX suppress ISC division. We hypothesize that suppression of ISC division and epithelial renewal may be a virulence strategy of *V. cholerae* designed to facilitate intestinal colonization.

**RESULTS**

The intestines of key and mtd^GOF mutants maintain their integrity during *V. cholerae* infection. We showed that a mtd^GOF mutant and key^1 and dredd*B118* IMD pathway null mutants were better able than control flies to resist oral *V. cholerae* infection (2, 3). Because studies utilizing electron microscopy suggested that the intestinal epithelium of control flies was breached during infection (3), we hypothesized that the increased tolerance of these mutants might arise from their ability to maintain the integrity of the intestinal epithelial barrier. To test this, we examined the intestinal adherens junctions of *V. cholerae*-infected control and mutant flies by immunofluorescence assay with a *Drosophila* E-cadherin (DCAD) distribution was evaluated in control flies (yw) and mtd^GOF, key, and dredd mutant flies. The E-cadherin protein was visualized with Alexa 568 (red). Nuclear DNA was stained with DAPI (blue). Scale bars: left, 50 μm; right, 20 μm.
ROS and thus confer tolerance to oxidative stress. 

Mtd and IMD pathway mutants are resistant to oxidative stress. Dual oxidase is the major source of ROS in the Drosophila intestine and has been shown to reduce the burden of Erwinia carotovora strain Ecc15 in the intestine (10). However, ROS can damage the host intestine if its production is unrestricted (11). Because the mammalian, mosquito, and yeast homologs of Mtd have been demonstrated to play a role in resistance to oxidative stress (20, 22–24), we hypothesized that mtdGOF and IMD pathway mutant flies (Fig. 1B to D). Thus, we hypothesized that the mtdGOF, key4, and dreddB118 mutants better tolerate V. cholerae infection because their intestinal epithelia are not subjected to or are better able to withstand the insult caused either directly by V. cholerae or indirectly by the intestinal innate immune response.

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ROS toxicity does not contribute to lethality in V. cholerae infection of the fly. Because mtdGOF and IMD pathway mutants were resistant to both V. cholerae infection and ROS, we questioned whether ROS generated in response to V. cholerae infection was lethal. To test this, we reduced ROS in flies both by ubiquitous overexpression of the immune-regulated catalase (IRC) and by knockdown of Duox transcription by RNAi (see Fig. S1A and C in the supplemental material) and monitored the effect on susceptibility to V. cholerae infection. These interventions did not prolong fly survival (see Fig. S1B and D). These results suggest that ROS production does not play a role in the death of V. cholerae-infected flies. Therefore, it is unlikely that Mtd and the IMD pathway alter susceptibility to V. cholerae infection by directly modulating intestinal ROS.

ROS production protects the fly against intestinal infection with Ecc15 by reducing the burden of bacteria (10, 28). Because decreased expression of dual oxidase also did not increase susceptibility to V. cholerae infection, we compared the susceptibility of V. cholerae to ROS with that of Ecc15. When harvested in early stationary phase, V. cholerae survived treatment with 32.5 mM H2O2, whereas Ecc15 did not survive exposure to 16.3 mM H2O2 (see Fig. S1E in the supplemental material). This resistance to ROS is correlated with the formation of bubbles upon exposure to H2O2 (see Fig. S1F), suggesting the generation of O2 by the action of bacterial catalase. Therefore, one possibility is that generation of H2O2 by dual oxidase does not protect the fly against V. cholerae infection because V. cholerae rapidly detoxifies this substance.

mtdGOF and IMD pathway null mutations increase ISC division in spite of similar burdens of commensal bacteria. In addition to the production of antimicrobial peptides and ROS, ISC division and epithelial remodeling are induced during intestinal infection, presumably to maintain epithelial integrity (29–32, 33, 34). Therefore, we hypothesized that the higher tolerance of Mtd gain-of-function (MtdGOF) and IMD pathway mutants to both ROS and intestinal V. cholerae might reflect increased rates of ISC division and therefore accelerated epithelial repair. To test this, we quantified the rates of ISC division in control and mutant flies. As shown in Fig. 3A, the absence of infection, both the mtdGOF and IMD pathway mutants had rates of ISC division that were at least three times as high as those of control flies. In contrast, mtdLOF alleles resulted in levels of ISC division lower than those of control flies (Fig. 3B). We hypothesize that increased expression of dual oxidase does not protect the fly against V. cholerae infection because V. cholerae rapidly detoxifies this substance. 

The synthesis of several antimicrobial peptides is decreased in IMD pathway mutants, while only dipteracin synthesis is decreased in the MtdGOF mutant (2, 35). We considered the possibility that the increased rates of ISC division observed in the IMD pathway and MtdGOF mutants were due to overgrowth of commensal bacteria in the absence of intestinal synthesis of the usual spectrum of antimicrobial peptides. To assess the burden of commensal bacteria, we measured the bacterial 16S rRNA gene levels
in uninfected control and mutant flies by quantitative PCR (qPCR) with universal primers. As shown in Fig. 3C, we found no significant difference in the 16S rRNA gene quantities in control and mutant flies. Therefore, we conclude that the overall burden of bacteria is not different between control flies and IMD pathway or Mtd\textsuperscript{GOF} mutants.

Repression of the IMD pathway in both progenitor cells and enterocytes increases ISC division; expression of Mtd increases ISC division only in progenitor cells. Asymmetric ISC division in the \textit{Drosophila} intestine gives rise to an ISC and an enteroblast, which differentiates most frequently into an enterocyte but may also differentiate into an enteroendocrine cell (36). Ubiquitous overexpression of the H isoform of Mtd (Mtd\textsubscript{RH}), which includes the Mtd TLDc domain but not the LysM domain, was previously shown to phenocopy the Mtd\textsuperscript{GOF} mutant, suggesting that this isoform is functional (2). To determine whether the IMD pathway and Mtd modulate ISC division locally from within intestinal progenitors and/or enterocytes, we expressed Mtd\textsubscript{RH}, mtd-RNAi, or key-RNAi in progenitor cells and enterocytes by using the esg-Gal4 and NP1-Gal4 drivers, respectively. Enterocytes greatly outnumber progenitor cells. Therefore, an interfering RNA expressed in progenitor cells would be expected to have a significant effect on transcription in the intestine as a whole only if progenitor cells are the major producers of the RNA target or the RNA persists through maturation. Expression of key-RNAi but not mtd-RNAi in progenitor cells had a significant effect on overall intestinal transcription (Fig. 4A and B). Because the IMD pathway is active in enterocytes, this suggests that key-RNAi may persist through maturation.

Expression of Mtd\textsubscript{RH} in progenitor cells significantly increased PH3 antibody staining in the midgut, while expression of mtd-RNAi had the opposite effect (Fig. 4C). However, expression of these constructs in enterocytes had no effect on PH3 antibody staining (Fig. 4D). These results suggest that Mtd expression within intestinal progenitors but not enterocytes activates ISC division.

key-RNAi increased PH3 antibody staining both when driven to progenitor cells and when driven to enterocytes (Fig. 4G and H). To establish that these observations were not unique to Key but rather reflected a property of the IMD pathway as a whole, we confirmed that ISC division was also increased by mutation of Relish and by rel-RNAi expression in progenitor cells and enterocytes (Fig. 4I). Because our data suggest that key-RNAi persists during enteroblast maturation, we hypothesize that the IMD pathway suppresses ISC division from within enterocytes. However, we cannot exclude an additional role for the IMD pathway in progenitor cells.

\textit{V. cholerae} suppresses ISC division. Because increased ISC division was correlated with decreased susceptibility to \textit{V. cholerae} infection, we hypothesized that \textit{V. cholerae} might suppress ISC division and epithelial renewal, as has previously been shown for \textit{Pseudomonas entomophila} (31, 32). Therefore, we correlated fly death with the number of cell divisions in the intestines of \textit{V. cholerae}-infected control flies as a function of time. As shown in Fig. 5A, ISC turnover decreased as flies ingested \textit{V. cholerae}, and this was correlated with host death. \textit{V. cholerae} infection also decreased ISC divisions in the intestines of mtd\textsuperscript{GOF} and key\textsuperscript{4} mutant flies. However, compared with those in control flies, ISC divisions remained elevated in these mutants (Fig. 5B). We therefore conclude that, in this infection model, epithelial renewal is jointly controlled by the host and the pathogen.

\textit{V. cholerae} has previously been shown to damage the intestinal epithelium. To rule out the possibility that decreased PH3 antibody staining in infected intestines reflected destruction of ISCs, we compared the numbers of ISCs in uninfected flies with those in flies infected with \textit{V. cholerae} by using a transgenic fly expressing green fluorescent protein (GFP) in ISCs. As shown in Fig. 5C in the supplemental material, there was no appreciable difference in the number of GFP-labeled cells after 48 h of infection.

CTX binds exclusively to progenitor cells and suppresses ISC division. CTX is a virulence factor in the \textit{Drosophila} model of infection (4). We therefore questioned whether CTX played a role in the suppression of ISC division. We first compared ISC division in flies fed either LB broth alone or LB broth inoculated with wild-type, \textit{A. escherichia} or \textit{A. escherichia} mutant. \textit{V. cholerae}, as shown in Fig. 6A and B, \textit{V. cholerae} \textit{A. escherichia} and \textit{A. escherichia} mutants colonized the fly intestine as well as wild-type \textit{V. cholerae} did but did not suppress ISC division as effectively. However, because ISC division
remained significantly suppressed in Drosophila infected with these mutants, we conclude that other bacterial factors must also contribute to this process.

To further establish the role of CTX in the suppression of ISC division, we fed flies increasing amounts of purified CTX and quantified the PH3+ cells. As shown in Fig. 6C, ingestion of CTX but not bovine serum albumin (BSA) at a concentration of 100 μM significantly decreased ISC proliferation. Our observations suggested to us that CTX might interact specifically with ISCs. To study this, we removed the intestines of esg-Gal4/GFP

![Graphs and images]

**FIG 4** Mtd and Key act in different cell types to regulate ISC proliferation. (A) Mtd levels in the intestines of uninfected control (esg-Gal4/+), esg>Mtd-RC, esg>Mtd-RH, and esg>mtd-RNAi flies. In comparison with the control, esg>Mtd-RC P = 0.0281, esg>Mtd-RH P = 0.0625, esg>MtdRNAi P = 0.4781 (*). (B) Mtd levels in the intestines of infected control (NP1-Gal4/+), NP1>Mtd-RC, NP1>Mtd-RH, or NP1>mtd-RNAi flies. In comparison with the control, NP1>Mtd-RC P = 0.0024 (*), NP1>Mtd-RH P = 0.0003 (*), NP1>MtdRNAi P = 0.0080 (*). (C) Enumeration of PH3 antibody-dependent immunofluorescence (PH3+) in the intestines of uninfected control (esg-Gal4/+), esg>Mtd-RC, esg>Mtd-RH, or esg>mtd-RNAi flies. In comparison with the control, esg>Mtd-RC P = 0.0024 (*), esg>Mtd-RH P = 0.0013 (*), esg>MtdRNAi P = 0.0099 (*). (D) Enumeration of PH3+ cells in the intestines of infected control (NP1-Gal4/+), NP1>Mtd-RC, NP1>Mtd-RH, or NP1>mtd-RNAi flies. In comparison with the control, NP1>Mtd-RC P = 0.4672, NP1>Mtd-RH P = 0.7877, and NP1>MtdRNAi P = 0.9099. (E) Key levels in the intestines of infected control (esg-Gal4/+), esg>Mtd-RC, esg>Mtd-RH, or esg>mtd-RNAi flies. In comparison with the control, esg>Mtd-RC P = 0.0002 (*), esg>Mtd-RH P = 0.0081 (*), esg>MtdRNAi P = 0.0407 (*).

**FIG 5** ISC proliferation is suppressed by V. cholerae. (A) Enumeration of cells exhibiting PH3 antibody-dependent immunofluorescence in the intestines of V. cholerae-infected yw flies over time correlated with fractional survival. (B) Enumeration of PH3+ cells in the intestines of yw (control [CTL]), mtdGOF, or key flies 48 h after V. cholerae ingestion. *: mtdGOF versus CTL, P < 0.005; key1 versus CTL, P < 0.01.
flies, whose intestinal progenitor cells express GFP, incubated these intestines with CTX, and visualized binding by immunofluorescence assay. As shown in Fig. 6D and E, the distribution of CTX closely overlapped that of GFP, indicating that CTX binds specifically to progenitor cells in the Drosophila intestine. A similar pattern of binding was observed after the incubation of intestines with the CTX-B subunit directly conjugated to a fluorescent dye (see Fig. S3 in the supplemental material). Fluorescence was greatly decreased by the addition of an excess of unlabeled CTX-B during incubation, which is consistent with a specific interaction of the B subunit of CTX (CTX-B) with ISC (see Fig. S3). We hypothesize that CTX binds to and acts specifically on intestinal progenitor cells to suppress proliferation.

DISCUSSION

The intestinal epithelium provides a physical barrier that enables the host to coexist with its intestinal microbiota, and epithelial renewal promotes host coexistence with these commensal bacteria by replacing enterocytes that are infected, intoxicated, or colonized with bacteria. Here we show that IMD pathway and mtdGOF mutants, which are resistant to intestinal V. cholerae infection and ROS ingestion, have increased rates of ISC division. This is not the result of an increase in the numbers of commensal bacteria, suggesting the possibility of direct regulation of epithelial regeneration by these pathways. Furthermore, we find that both V. cholerae and CTX suppress ISC proliferation. We propose that increased basal rates of intestinal epithelial renewal counter suppression of ISC division by V. cholerae, thus providing protection against infection.

The IMD pathway and the Mtd protein modulate both AMP synthesis and intestinal regeneration. We considered the possibility that the relationship between AMP synthesis and ISC division is indirect. In the absence of AMP synthesis, proliferation of commensal bacteria could lead to enterocyte damage and subsequent epithelial regeneration. However, we did not observe an increase in the population of commensal bacteria in the intestines of IMD pathway and MtdGOF mutant flies. AMP synthesis is quite low in the uninfected Drosophila intestine because of multiple regulators that repress the transcription of the genes that encode these proteins (37, 38). Our findings suggest that, in the absence of infection, AMPs do not exert significant pressure on the commensal microbiota. Instead, we hypothesize that AMP synthesis and ISC division are coregulated by the IMD pathway and Mtd, thus orchestrating a coordinated response to a breach in the intestinal

FIG 6 ISC proliferation is suppressed by CTX. (A) Bacterial burdens of flies infected with wild-type V. cholerae (WT), a ΔctxA mutant, or a ΔctxB mutant for 48 h. Measurements were not statistically significantly different. (B) Enumeration of cells exhibiting PH3 antibody-dependent immunofluorescence (PH3+) in the intestines of yw flies 48 h after infection with wild-type V. cholerae, a ΔctxA mutant, or a ΔctxB mutant. In comparison with wild-type V. cholerae, ΔctxA mutant P = 0.0066 (*), and ΔctxB mutant P = 0.0036 (*). (C) Enumeration of PH3+ cells in the intestines of yw flies fed PBS, purified CTX solution (0.1 mg/ml), or BSA (1 mg/ml). CTX versus PBS, P = 0.0027 (*), CTX versus BSA, P = 0.0318 (*). (D and E) Immunofluorescent visualization of CTX binding to the ISCs of esg-Gal4>GFP flies. ISCs and enteroblasts were labeled with GFP, and CTX was detected with an anti-CTX antibody conjugated to Alexa 546 (red). Nuclear DNA was stained with DAPI (blue). Scale bars: D, 50 μm; E, 20 μm.
barrier. However, proof of this hypothesis requires further experimentation.

Intestinal bacteria have previously been shown to modulate epithelial regeneration in Drosophila (29, 31, 39, 40). Here we show that infection with V. cholerae suppresses ISC division. V. cholerae colonizes the crypts of the human terminal ileum, where ISCs are found. Because V. cholerae has access to stem cells, this process could also occur in the human intestine. We propose that arrest of epithelial renewal in the mammalian intestine would facilitate colonization by V. cholerae.

CTX is, in part, responsible for the arrest of ISC division by V. cholerae. Selective adherence of CTX to progenitor cells within the intestine suggests a direct effect on these cells. While suppression of ISC division is a novel function for CTX, it is not surprising that cellular intoxication would result in changes in cellular physiology that extend beyond the osmotic diarrhea observed in mammals. Many questions remain regarding the mechanism by which CTX acts on arthropod ISCs. CTX is an A1B5 toxin (41). The B subunit is responsible for adhesion to the CTX receptor, GM1, a ganglioside that is present on the surface of mammalian intestinal epithelial cells. While gangliosides are present in the membranes of Drosophila cells (42, 43), it is not certain that the canonical CTX receptor GM1 or another lipid with a similar head group is present in the membranes of Drosophila cells. If CTX enters the cell by a different route or does not enter at all, its mechanism of action may be distinct from that which has been delineated in mammalian cells.

Unlike E. carotovora and P. entomophila, which are plant pathogens (44, 45), V. cholerae is a noninvasive intestinal pathogen of humans. Interference with intestinal epithelial regeneration is a novel virulence mechanism for V. cholerae. Because humans remain the only adult mammals that are known to become colonized with V. cholerae in the absence of chemical or surgical manipulation, corroboration of our observation in mammals must await the development of an appropriate mammalian model. However, we propose that suppression of ISC division may be used by V. cholerae as a strategy to enhance colonization of the epithelial surface. From our studies and those of others, a model is emerging in which active regulation of intestinal epithelial renewal by both commensal and pathogenic microbes determines the extent of the epithelial colonization, disintegration, and invasion that characterize the interaction of each of these classes of microbes with the intestinal epithelium.

MATERIALS AND METHODS

Fly strains and media.yw flies were used as controls unless otherwise noted. The dreddB118 mutant and eg-Gal4 driver were gifts of Norbert Perrimon (Harvard Medical School, Boston, MA). The key1 mutant was a gift of Neal Silverman (University of Massachusetts Medical Center, Worcester, MA). Won Jae Lee generously provided us with the NPI-Gal4 driver. The mtdE06035, mtdE06038, mtdE06039, and Daughterless-Gal4 strains were obtained from the Bloomington Stock Center. The mtdE06035 and mtdE06038 mutant strains were obtained from the Harvard Exelixis Collection. The UAS-key-RNAi and UAS-MtdRNAi mutant strains were obtained from the Vienna Drosophila RNAi Center. After arrival in our lab, all Drosophila strains were maintained at 24°C on our standard Drosophila medium for several generations prior to use in these experiments. The standard fly medium used contained yeast, soy flour, cornmeal, malt, corn syrup, and agar. Propionic acid and Tegosept (a Drosophila antifungal agent also known as 4-hydroxybenzoic acid methyl ester, methyl 4-hydroxybenzoate, and Nipagin) were added for control of microbial growth.

Bacterial strains and growth media. V. cholerae serogroup O139 strain MO10 and E. carotovora strain Ecc15 were propagated overnight in LB broth (Fisher) at 27°C prior to use in infection assays (45, 46).

Infection susceptibility assays. Survival upon intestinal infection was assayed as previously described (2, 4). Briefly, an overnight bacterial culture that had reached early stationary phase was diluted in a 1:10 ratio in fresh LB broth. Two milliliters of this suspension was added to a cellulose acetate plug positioned at the bottom of a fly vial. Thirty male or female flies, as indicated, were divided evenly among three vials prepared in this fashion to yield three independent experimental replicates. Viable flies were counted at least once in every 24-h period. Survival curves were constructed, and log-rank analysis incorporating results from each of the three independent experiments was used to determine statistical significance. The reproducibility of all survival data was confirmed in at least one additional experiment performed in triplicate on a different day with flies obtained from different food vials.

Assays of bacterial and insect susceptibility to H2O2. For experiments evaluating the resistance of flies to H2O2, a 9.8 M solution of stabilized H2O2 (Sigma) was diluted with a 5% sucrose solution to yield a 196 mM solution of this agent. Two millennials of this preparation was used to saturate a cellulose acetate plug. Flies were transferred to vials containing a freshly prepared H2O2 solution daily throughout the course of the experiment. Resistance of bacteria to H2O2 was assayed as follows. Three independent cultures of the bacterial strains referenced were grown to stationary phase. Increasing concentrations of H2O2, as indicated, were added to each culture. The cultures were allowed to stand for 5 min, tubes were photographed, and then 10 μl of the treated cultures was spotted onto LB agar plates. All assays were performed with three independent cultures and repeated with similar results.

RT-qPCR assays. Flies were homogenized in TRIzol reagent (Invitrogen). Total RNA was extracted once, treated with DNase I, and then extracted a second time. One microgram of purified RNA was used as a template for the synthesis of cDNA with a QuantiTect reverse transcription (RT) system (Qiagen). The resulting cDNA was used for qPCR with iTaq SYBR green Supermix with carboxy-X-rhodamine (Bio-Rad) and 2 pmol of the relevant primers in a 20-μl reaction volume. The experiments were conducted with a StepOnePlus PCR system (Applied Biosystems). Transcript levels of each gene were quantified by comparison with a standard curve and normalized to the level of the reference gene rp49. The sequences of the primers used are available upon request.

Quantification of commensal bacteria by qPCR. Freshly eclosed flies were either transferred to fresh vials or maintained in the same vials for 3 to 5 days. They were then washed with 70% ethanol and phosphate-buffered saline (PBS) plus 0.1% Tween 20 to remove external bacteria. Genomic DNA was isolated from whole flies, intestines only, or bacteria with a Wizard genomic DNA purification kit (Promega). The following previously validated universal primer set was used to detect the 16S rRNA gene from all of the bacterial species present in the samples (47): F, GCC GCCGTAATTAGTAAGGA; R, CGTACCCCTTTACCAGCGGAATG. Absolute numbers of bacteria were calculated from a standard curve generated with DNA isolated from a known quantity of Escherichia coli cells as a template for qPCR.

Immunofluorescence assays. For immunofluorescence assays, infections were carried out for 48 h prior to preparation and imaging unless otherwise noted. Fly tissues were dissected in PBS, transferred immediately to 4% formaldehyde in PBS, and incubated for 30 min at room temperature. Tissues were then rinsed three times for 30 min each in PBS and dehydrated in a graded series of ethanol solutions. Tissues were then embedded in paraffin and sectioned at 7 μm. Sections were mounted on slides and stained with hematoxylin and eosin. The sections were then treated with 0.1% Triton and 1% BSA and incubated overnight at 4°C in PBS containing rabbit anti-CTX (1:300; Sigma), or rabbit anti-PH3 (1:2,000; Millipore) antibody. After rinsing, a second overnight incubation was performed with the Alexa 568-conjugated goat anti-rat IgG (1:200; Invitrogen), Alexa May/June 2013 Volume 4 Issue 3 e00337-13
488-conjugated goat anti-mouse IgG (1:100; Invitrogen), Dylight 549-conjugated goat anti-mouse IgG (1:100; Jackson ImmunoResearch), or Alexa 546-conjugated goat anti-rabbit IgG (1:200; Invitrogen) secondary antibody. The tissues were rinsed again with the addition of 4’,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) to the last wash. Tissues were mounted in Vectashield and examined with either an Eclipse TE-2000-E phase-contrast microscope (Nikon) for enumeration of ISC mitoses or an LSM700 confocal microscope (Zeiss) for image collection.

For staining with labeled CTX, the dissected intestines were incubated with 20 μg/ml of CTX-B conjugated to Alexa Fluor 594 (Invitrogen) in Grace’s insect medium for 30 min at 4°C and then washed and visualized. To test specificity, Alexa Fluor 594-conjugated CTX-B was incubated with intestines in the presence of a 10-fold excess of unlabelled CTX-B.

For convenience, PH3 antibody staining was performed with the in-testines of wild-type and mutant female flies, which have higher rates of ISC division than those of males but similar resistance patterns (see Fig. S4A and B in the supplemental material). Using our experimental design, we found that the rates of stem cell division measured in the intestines of uninfected control flies were somewhat higher than previously reported. To determine whether our experimental design, which utilizes LB broth rather than 5% sucrose, might be responsible for this, we measured ISC divisions in control yw female flies fed standard fly food, LB broth, or a 5% sucrose solution. ISC divisions were approximately five times as low in flies fed 5% sucrose as in those fed standard fly food or LB broth (see Fig. S4C). This is consistent with the observation that ingestion of nutrient-rich growth medium results in increased ISC divisions (48). Strain Eccl15 is known to greatly activate ISC division (29, 49–51). To establish that we could reproduce this result by using our experimental design, we fed Drosophila flies strain Eccl15 in LB broth. Intestinal mitoses increased by a factor of 2.5 when flies were fed Eccl15 bacteria for 24 h (see Fig. S4C). Therefore, we conclude that while basal rates of ISC division are higher in our experimental model, the response to intestinal bacteria is similar to that observed in other experimental designs.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.00337-13/-/DCSupplemental.

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