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Homeostatic Epithelial Renewal in the Gut Is Required for Dampening a Fatal Systemic Wound Response in *Drosophila*

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SUMMARY

Effective defense responses involve the entire organism. To maintain body homeostasis after tissue damage, a systemic wound response is induced in which the response of each tissue is tightly orchestrated to avoid incomplete recovery or an excessive, damaging response. Here, we provide evidence that in the systemic response to wounding, an apoptotic caspase pathway is activated downstream of reactive oxygen species in the midgut enterocytes (ECs), cells distant from the wound site, in *Drosophila*. We show that a caspase-pathway mutant has defects in homeostatic gut cell renewal and that inhibiting caspase activity in fly ECs results in the production of systemic lethal factors after wounding. Our results indicate that wounding remotely controls caspase activity in ECs, which activates the tissue stem cell regeneration pathway in the gut to dampen the dangerous systemic wound reaction.

INTRODUCTION

Epithelial cells, including gut cells and hair cells, undergo turnover in mammals (Slack, 2000). Although tissue-specific stem cells have not been discovered in most tissues in *Drosophila*, the gut does possess them and undergoes constant cell turnover; therefore, it is often used to investigate the physiological functions of epithelial cell turnover (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Epithelial cell turnover is thought to be indispensable because these cells are often exposed to direct stress from the environment and can be easily damaged (Buchon et al., 2009a; Cliffe et al., 2005; Slack, 2000). Although the epithelia of tissues such as the trachea and epidermis, unlike those of the midgut and malpighian tubule, do not appear to undergo self-renewal in *Drosophila* (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Singh et al., 2007), they are nonetheless maintained. Unlike epithelial cells that are protected by cuticle, the gut cells in *Drosophila* are exposed to the external environment, and the cell renewal observed in the gut may be in response to environmental insults. However, even though the cells of the malpighian tubule are not exposed directly to the external environment, they still undergo cell turnover. This raises the possibility that epithelial cell turnover has functions in addition to maintaining tissue homeostasis by removing epithelial cells that suffer direct damage from the environment.

In the gut of young healthy *Drosophila*, the cell number is tightly regulated by intestinal stem cell (ISC) proliferation and cell death (Biteau et al., 2008). Recent studies revealed that the mechanism of gut cell turnover is quite similar between *Drosophila* and mammals (Biteau et al., 2011; Casali and Batlle, 2009). During cell turnover in the *Drosophila* midgut, which is functionally equivalent to the mammalian small intestine, ISCs can divide asymmetrically into an ISC and an enteroblast (EB), or symmetrically into two ISCs or two EBs (de Navascués et al., 2012). An EB can differentiate into either an enteroendocrine cell (EE) or an enterocyte (EC) (Figure 1A) (de Navascués et al., 2012; Micchelli and Perrimon, 2006; O'Brien et al., 2011; Ohlstein and Spradling, 2006). ECs, which are huge, absorptive cells, and EEs, which are secretory cells, undergo cell death and are replaced by new ECs and EEs. Previous reports on *Drosophila* have shown that, after direct damage to the gut epithelia by oxidative stress, infection by harmful bacteria, or ingestion of a toxic compound, ISCs proliferate rapidly to supply new ECs (Amcheslavsky et al., 2009; Buchon et al., 2009b; Chatterjee and Ip, 2009; Jiang et al., 2009; Shaw et al., 2010). An increase in caspase-activated cells is also observed after midgut damage, suggesting that the ECs undergo apoptosis (Buchon et al., 2009b). These damaged ECs produce cytokines, such as Unpaired (Upd), to accelerate ISC proliferation in a manner similar to the mechanism of compensatory proliferation observed during tissue repair (Buchon et al., 2009b; Jiang et al., 2009).

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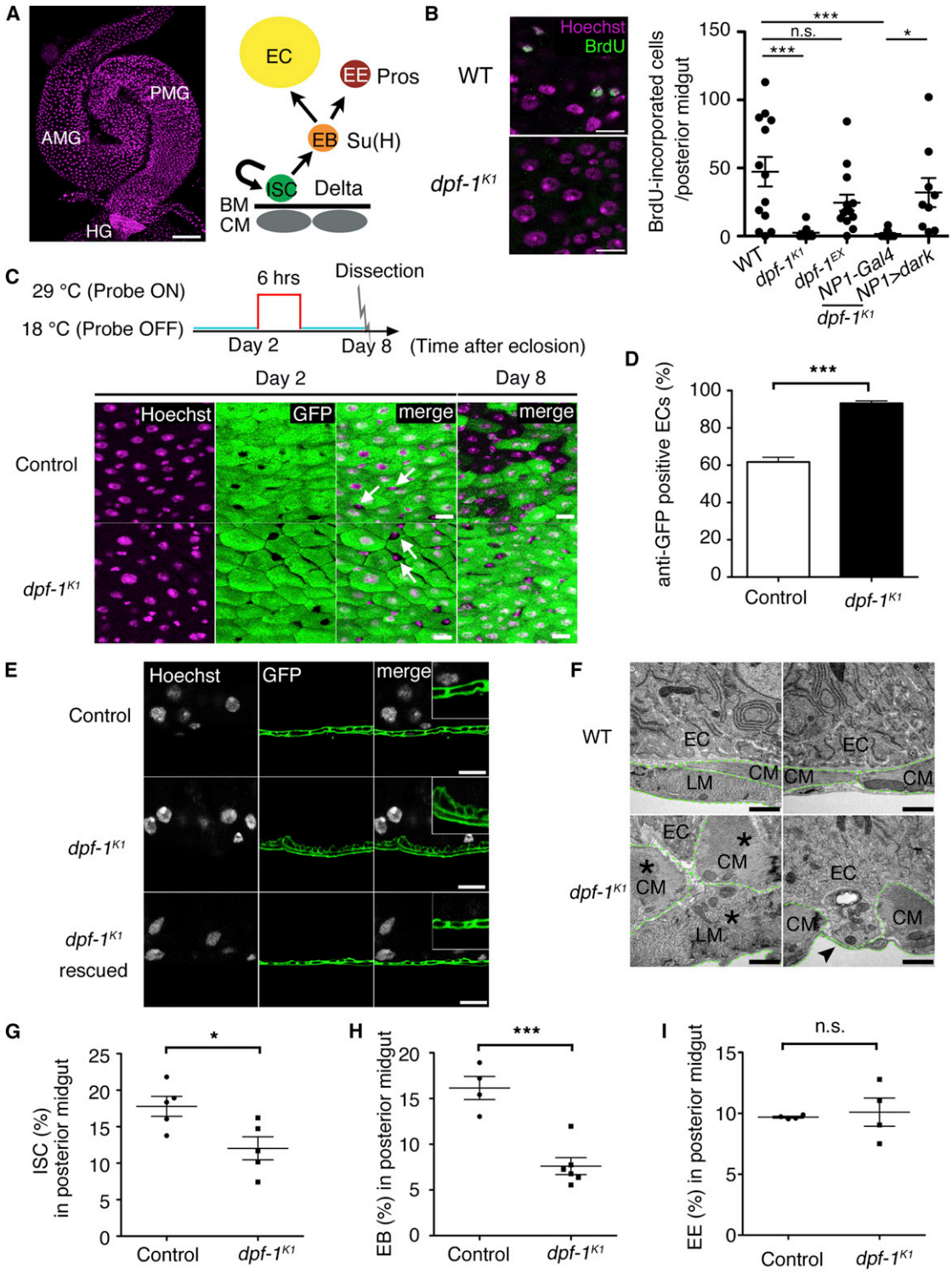

Figure 1. *dpf-1*^{K1} Has Defects in Gut Epithelial Turnover and Structure

(A) Morphological feature of the *Drosophila* midgut (left) and a schematic drawing for the self-renewal process of midgut cells at the adult stage (right). All of the immunohistochemical and EM analyses in this study were performed on the posterior midgut. ISCs proliferate to generate ISCs and EBs, which differentiate into ECs or EE cells (right). Markers for each cell type: Delta for ISC, Su(H) for EB, and Pros for EE (right). AMG, anterior midgut; PMG, posterior midgut; HG, hindgut; magenta, Hoechst (left). Scale bar, 100 μm.

(B) BrdU incorporation. Left: Magenta, Hoechst; green, BrdU labeling. Scale bars represent 10 μm. Right graph: The number of BrdU-incorporated cells in each gut. WT, wild-type.

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Gut cell turnover is regulated by a balance between cell death and stem cell proliferation (Jiang et al., 2009). We previously identified a hypomorphic mutant of the caspase activator *dapaf-1/dark/HAC-1* (Kanuka et al., 1999). Here, we found that this *dark* mutant had impaired homeostatic gut cell renewal, and we used it to analyze the biological significance of homeostatic epithelial renewal. The mutant flies were viable and fertile, suggesting that homeostatic epithelial renewal is dispensable under normal culture conditions. However, the *dark* mutant was sensitive to the systemic wound response. We found that a wound distant from the gut in wild-type flies induced caspase activation in the ECs of the gut downstream of reactive oxygen species (ROS). These caspase-activated cells were removed from the gut cell layer to induce gut cell renewal. We showed that caspase inhibition in the ECs prevented gut cell turnover, thereby introducing a lethal systemic factor into the hemolymph after wounding. Thus, homeostatic gut epithelial renewal plays a crucial buffering role in reducing the effect of gut sensitivity to the wound response and in maintaining body homeostasis.

RESULTS

Caspase Activity Is Required for Gut Cell Turnover

To investigate whether cell death is required for gut cell turnover and homeostatic maintenance of the gut in adult *Drosophila*, we used a caspase pathway mutant to prevent the cell death of ECs and EEs. Several apoptosis-executing caspases, such as DCP-1 and drICE, have been identified in *Drosophila* and are functionally redundant (Xu et al., 2006). We therefore used a mutant of the caspase activator *dapaf-1/dark/HAC-1* (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). The null mutant of *dark* shows pupal lethality (Mills et al., 2006; Srivastava et al., 2007b), so we used a hypomorphic mutant, *dpf-1^{K1}*, which has a P-element insertion in the 5′-UTR of the *dark* gene (Kanuka et al., 1999). Although cell death and caspase activity are suppressed during development in *dpf-1^{K1}* compared with wild-type, the flies survive to adulthood (Kanuka et al., 1999).

We first examined whether the spontaneous gut cell turnover of the *dpf-1^{K1}* midgut was suppressed, using bromodeoxyuridine (BrdU) incorporation. BrdU is incorporated into proliferating ISCs and endoreplicating EBs that differentiate into polyploid ECs. Both ISC proliferation and EB endoreplication were significantly reduced in the *dpf-1^{K1}* flies (Figure 1B). The decreased number of BrdU-positive cells was rescued by the overexpression of *dark* in ECs by an EC-specific driver, *NP1-Gal4* (*Myo1A-Gal4*) (Figures 1B, right, and S1A–S1E) (Jiang et al., 2009; Karpowicz et al., 2010; Poernbacher et al., 2012; Ren et al., 2010). This decrease was also rescued in a line in which the P-element of *dpf-1^{K1}* was precisely excised (Figure 1B, right). These data support the idea that flies have a defect in gut cell turnover due to the inhibition of gut EC death (see Figure 3D).

To investigate the gut EC turnover rate, we used flies that transiently express a fluorescent protein in the ECs upon heat shock (Figure 1C, top). We used the CD8::PARP::Venus probe as a stable fluorescent protein in this experiment to allow us to mark EC cells with the GFP antibody or CD8 antibody, although this probe is generally used to observe caspase activation with the cleaved PARP antibody (as described in Figure 3). The staining patterns of GFP and CD8 in the ECs were identical (Figure S1F), and these staining patterns were not affected by caspase activation (Figure S1G). Because the fluorescence of CD8::PARP::Venus is very stable, the GFP antibody-positive ECs remained detectable unless they underwent turnover. Six hours after adult flies were heat shocked to express the probe, all of the ECs of the control and *dpf-1^{K1}* flies were stained with the GFP antibody (Figure 1C, bottom). Six days after the transient expression of this probe, we dissected the fly gut and observed the remaining GFP-positive ECs. In the controls, approximately 60% of the ECs were positive for GFP, but in *dpf-1^{K1}* more than 90% of the ECs were GFP positive, indicating that *dpf-1^{K1}* has a defect in EC turnover (Figure 1D). Collectively, these results suggest that *dpf-1^{K1}* has defects in homeostatic gut cell turnover, and that caspase activity is required to maintain homeostatic renewal.

Midgut Structure Is Disrupted in *dpf-1^{K1}*

To investigate the architecture of the midgut in *dpf-1^{K1}*, we visualized the basement membrane (BM) structure that surrounds the muscle by using collagen IV-GFP (Srivastava et al., 2007a). In the *dpf-1^{K1}* gut, the two layers of collagen IV-GFP signal were farther apart and the cells were more rounded than in the control (Figure 1E). A similar disorganization of the BM is observed in dextran sulfate sodium (DSS)-mediated EC injury (Amcheslavsky et al., 2009), a disease model of colitis, suggesting that the midgut cells were damaged in *dpf-1^{K1}*. The BM phenotype in *dpf-1^{K1}* was rescued by *dark* overexpression in ECs (Figure 1E). Flies overexpressing the pan-caspase inhibitor *p35* in ECs also showed the disrupted collagen IV-GFP signal (data not shown). We further examined the defects in the muscle layer by electron microscopy (EM). In the *dpf-1^{K1}* gut, structural changes in the circular muscle (CM) and longitudinal muscle (LM), such as rounder and taller cells (Figure 1F, asterisks), were frequently observed, as was the occasional absence of

(C and D) *dpf-1^{K1}* has defects in EC turnover at the adult stage.
(C) Experimental procedure (top) and EC turnover (bottom). GFP-negative cells with small nuclei (arrows) were EEs. Magenta, Hoechst; green, GFP. Scale bars represent 10 μm.
(D) Percentage of GFP-positive ECs 6 days after the temporary expression of CD8::PARP::Venus.
(E) BM visualization by collagen IV detection in sagittal sections of the posterior midgut by confocal microscopy. White, Hoechst; green, GFP (for collagen IV-GFP). Scale bars represent 10 μm.
(F) Electron micrographs of the gut. BMs are indicated with green dashed lines. Top: Lumen side. The CM and LM cells in *dpf-1^{K1}* were rounder and taller than those of the WT (asterisks), and the muscle cells between the ECs and body cavity were occasionally absent in *dpf-1^{K1}* (arrowhead). Scale bars represent 1 μm.
(G–I) Proportion of gut cell types. Percentage of (G) Dl-lacZ-positive cells (ISCs), (H) Su(H)-lacZ-positive cells (EBs), and (I) Pros-positive cells (EEs) in the posterior midgut. The number of cells that were positive for each marker was counted and calculated as a percentage of the Hoechst-positive cells.
Error bars in all graphs indicate SEM.

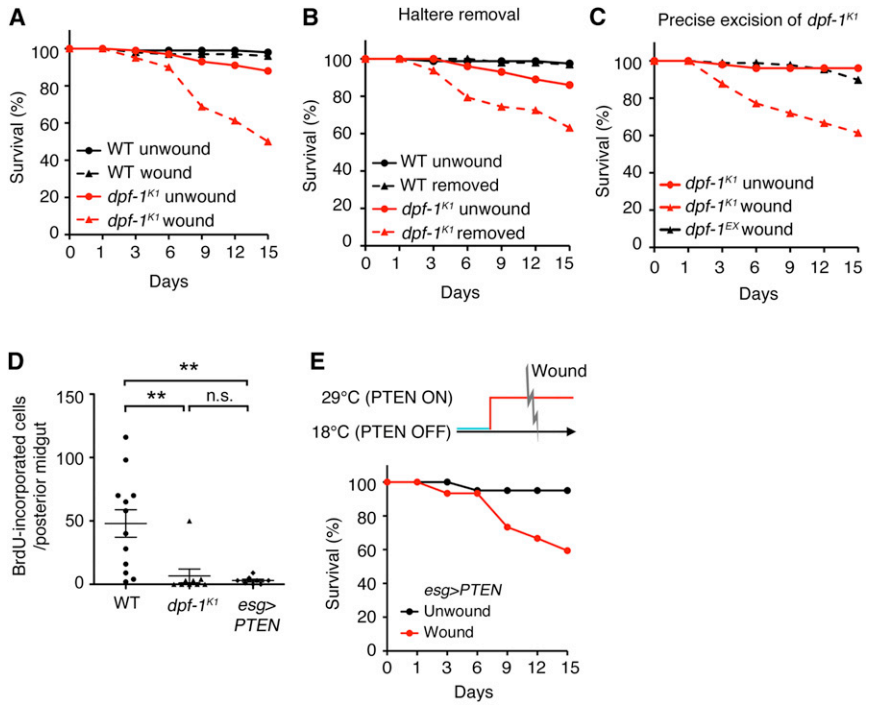

Figure 2. *dpf-1*^{K1} Flies Are Sensitive to Wounding

(A–C) Survival rate of wounded flies. Flies 2 days after eclosion were pricked on the abdomen with a microinjection needle, or halteres were removed. Day 0 is the day of wounding or removing. Unwounded flies were used as a control. (A) Wounding. (B) Haltere removal. (C) Wounding of the *dpf-1*^{EX} fly line.

(D) Number of BrdU-incorporating cells in the posterior midgut. *PTEN* was overexpressed in flies from 2 days after eclosion by shifting the temperature from 18°C to 29°C. BrdU was administered in the food for 3 days from 6 hr after the temperature shift to 29°C. Error bars in the graph indicate SEM.

(E) Top: Experimental procedure. *PTEN* expression was induced in the ISCs from 6 hr before wounding. Bottom: Survival rate after wounding.

See also Figures S2 and S3.

muscle cells between the ECs and the body cavity (Figure 1F, arrowhead).

Because the gut cell turnover and gut structure were altered in *dpf-1*^{K1}, we investigated whether the proportion of gut cell types in this mutant was different from that in wild-type flies. To examine this possibility, we performed immunohistochemistry with cell-type-specific markers (Figure 1A). The percentages of ISCs (*Dl-lacZ*, *lacZ*-expressing cells) and EBs (*Su(H)-lacZ*, *lacZ*-expressing cells) were lower in *dpf-1*^{K1} than in the control (Figures 1G and 1H). However, the number of EEs (Prospero-positive cells) did not differ significantly between *dpf-1*^{K1} and wild-type (Figure 1I). These midgut phenotypes suggested that a defect in ISC division led to the reduced ISC and EB levels, and that caspase-mediated gut cell turnover is critical for maintaining the gut architecture under normal culture conditions.

dpf-1^{K1} Is Sensitive to Wounding

dpf-1^{K1} is a viable mutant that is defective in gut epithelial renewal. This mutant was similar to the wild-type in appearance, body weight (Figure S2A), and food intake (Figure S2B), and the adults were fertile; therefore, gut epithelial renewal does not seem to be essential under normal culture conditions. We next asked whether gut epithelial renewal is required for survival under stress. Previous studies suggested that gut cell turnover is accelerated to maintain homeostasis after direct tissue damage to the gut (Amcheslavsky et al., 2009; Chatterjee and Ip, 2009), and our results from DSS feeding experiments indicated that *dpf-1*^{K1} is more sensitive to direct gut damage (Figure S3A). We then asked whether the stress induced by other tissue damage affects *dpf-1*^{K1} survival. To elicit tissue-damage-induced stress under normal culture conditions without directly injuring gut cells, we wounded the fly cuticle by pricking it with a microinjection needle. This protocol induces systemic stress as a wound response. The scab formation was similar between wild-type and *dpf-1*^{K1} flies (Figure S2C); however, the *dpf-1*^{K1} flies died after wounding (Figure 2A), indicating that *dark* might contribute to the flies' response to wounding. To check the generality of the *dpf-1*^{K1} injury-response phenotype, we removed the halteres of *dpf-1*^{K1} flies and again observed the wound-induced lethal phenotype (Figure 2B). The wound-induced lethality was fully rescued by the precise excision of the P-element of *dpf-1*^{K1}, confirming that this phenotype was not due to the *dpf-1*^{K1} genetic background (Figure 2C). We also performed the pricking experiment under sterile conditions and confirmed that the death of *dpf-1*^{K1} was not due to bacterial infection (Figure S3B).

We then investigated whether ISC proliferation, which is important for gut cell renewal, was required for the viability of flies after wounding. *PTEN* overexpression is reported to negatively regulate the cell division of proliferative histoblasts (HBs) and cells in the eye during development (Huang et al., 1999; Nakajima et al., 2011). By using the *esg-Gal4* driver to express a reporter protein in proliferating cells after adult eclosion (*esg-Gal4* driver with *tub-Gal80*^{ts}), we restricted the expression pattern to gut cells (Figure S1). To avoid a developmental effect of overexpressing *PTEN*, we used a fly line that overexpresses *PTEN* under *esg-Gal4* starting 2 days after adult eclosion. Using BrdU incorporation, we confirmed that the ISC proliferation in the midgut was suppressed in the flies that expressed *PTEN* (Figure 2D). The *PTEN*-expressing flies were, however, susceptible to wound-induced lethality (Figure 2E), supporting the idea that gut cell turnover is required to overcome wound stress.

Caspase Is Activated in ECs after Wounding

ISC proliferation and gut cell turnover are stimulated when caspase-mediated EC death is induced by DSS feeding, bacterial infection, or the expression of *reaper* (cell death inducer via caspase activation; Amcheslavsky et al., 2009; Buchon et al., 2009a; Jiang et al., 2009). Thus, it was possible that the wound caused

gut cell turnover by activating caspase in ECs. To examine this possibility, we observed caspase activation in the gut after wounding. To detect caspase activity in the gut, we performed imaging analysis on flies expressing the caspase indicator SCAT3, which is a fluorescence resonance energy transfer (FRET)-based indicator that enables caspase-3-like DEVDase activation to be monitored by a decrease in the FRET ratio (Takemoto et al., 2003). Using a fly that overexpresses SCAT3 ubiquitously (Nakajima et al., 2011), we first investigated whether caspase was activated in the gut after pricking. In the midgut, caspase was strongly activated in some ECs with large nuclei, as early as 30 min after wounding (Figure 3A). We also tested another tissue, the fat body, which is located between the cuticle and the gut, but found no caspase activation after wounding (Figure S4).

To further identify the caspase-activated cells, we used flies expressing CD8::PARP::Venus, a probe used for immunohistochemical analyses of caspase activity, with an antibody against cleaved PARP (cPARP), to identify caspase-activated cells (Figure S5A) (Florentin and Arama, 2012; Lee et al., 2011; Rumpf et al., 2011; Schoenmann et al., 2010; Williams et al., 2006). The cPARP-positive cells were negative for Delta (ISC marker) and Pros (EE marker) (Figures 1A, 3B, and 3C). On the other hand, in flies that overexpressed CD8::PARP::Venus in the ECs, cPARP-positive cells were observed in the midgut (Figure 3D). The cPARP-positive ECs were observed within 30 min after wounding, consistent with the result from the SCAT3 experiment, and were still observed even 24 hr after wounding (Figure 3D). The cPARP-positive cells were not observed in *dpf-1*^{K1}, confirming that the cPARP antibody detects caspase activation in ECs after wounding (Figure 3D). Furthermore, no cPARP-positive cells were detected in the fat body or epidermis (Figure S5B). We also detected no cPARP signal in the brain (Figure S5B). The cPARP-positive ECs were not clustered but rather were scattered throughout the entire midgut, suggesting that the wound triggers the response in the ECs throughout the midgut.

We also tested the caspase activation in ECs of the fly with an ISC proliferation defect. When ISC proliferation was inhibited by *PTEN* overexpression (Figure 2D), caspase activation was severely inhibited, suggesting that a mutual interaction between ISC proliferation and EC cell death is crucial for the systemic wound response (Figure S5C).

ROS Mediate the Caspase Activation in ECs after Wounding

Wounding induced caspase activity in the midgut, an organ distant from the wounded site, suggesting that a wound on the body could impact gut homeostasis. It was previously reported that hemocytes monitor body homeostasis (Babcock et al., 2008), and that the hemocyte-derived serine protease Hayan, which converts prophenoloxidase to phenoloxidase, is activated to produce H_2O_2 in the hemolymph as a systemic wound response (Nam et al., 2012). When the fly cuticle is injured, ROS induce melanization at the wound site, which prevents hemolymph loss and kills locally invading bacteria (Nosanchuk and Casadevall, 2003; Wang et al., 2010). In addition, ROS are reported to mediate ISC proliferation in *Drosophila* during the defense response against gut damage caused by aging or pathogenic bacteria (Buchon et al., 2009b; Hochmuth et al., 2011; Wang et al., 2003).

We therefore investigated the contribution of ROS to the caspase activation in ECs after wounding. Administration of the antioxidant N-acetylcysteine (NAC) in the food (Buchon et al., 2009a; Nam et al., 2012) decreased the number of cPARP-positive cells in the midgut of the wounded fly, suggesting that ROS function to activate caspase in the ECs after wounding (Figure 3E). We then asked whether caspase activation after injury depends on Hayan, a hemocyte-derived serine protease. The number of cPARP-positive cells did not decrease in the *Hayan* mutant (Figure 3F), indicating that caspase activation through ROS is independent of Hayan. We next investigated the possibility that ECs are a source of the ROS that act upstream of caspase activation. The number of cPARP-positive cells was decreased by the overexpression of an antioxidant enzyme, SOD1, driven by EC-specific *NP1-Gal4* (Figure 3F). Furthermore, knocking down NADPH oxidase 1 (*NOX1*) decreased the number of cPARP-positive cells after injury (Figure 3F). These findings suggested that when the epithelium is wounded, ECs generate ROS to activate caspase, which in turn induces gut cell turnover. Since melanization at the wound site was the same in wild-type and *dpf-1*^{K1} flies (Figure S2C), the redox signaling from the wound site to the hemolymph may be the same in *dpf-1*^{K1} as in wild-type flies. NAC-fed flies are sensitive to wounding, and ROS-mediated activation of neuronal c-Jun N-terminal kinase (JNK) is required to protect them from wound-induced death (Nam et al., 2012). In addition to this JNK-mediated mechanism, our data indicate that ROS-mediated signaling, including caspase activation in ECs, is required for the systemic wound response.

In addition to ROS, Upd is induced after tissue damage and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is activated to stimulate hemocyte proliferation (Agaisse et al., 2003; Pastor-Pareja et al., 2008). Also, direct gut damage by an oral bacterial infection or toxic chemical causes the production of the secreted mediator Upd3 in ECs (Buchon et al., 2009b). Thus, together with ROS, *upd3* may be activated and contribute to ISC proliferation even when a wound is distant from the gut. We observed that *upd3* was induced in the gut and near the wound site within 24 hr after wounding in both wild-type and *dpf-1*^{K1} (Figure S6). Thus, in addition to caspase activation, humoral factors that stimulate ISC proliferation are rapidly upregulated after wounding.

Caspase-Activated ECs Undergo Cell Death

To gain insight into the fate of the caspase-activated cells, we checked the position and morphology of the cPARP-positive ECs. To visualize the gut cell layer, we stained the luminal side of the gut cells and the muscle cells with phalloidin and cPARP antibody (Figure 3G). We observed some cPARP-positive ECs inside the gut cell layer (Figure 3G, arrow) and some that were emerging from the gut cell layer (Figure 3G, arrowheads, and Figure S5D). In addition, some of the cPARP-positive cells did not show staining with the nuclear dye Hoechst 33342 (Figure 3G, arrowheads). The EM image shows a cell with a faint nuclear membrane emerging from the gut cell layer at the luminal side (Figure 3H, arrowhead). These delaminated ECs appeared to

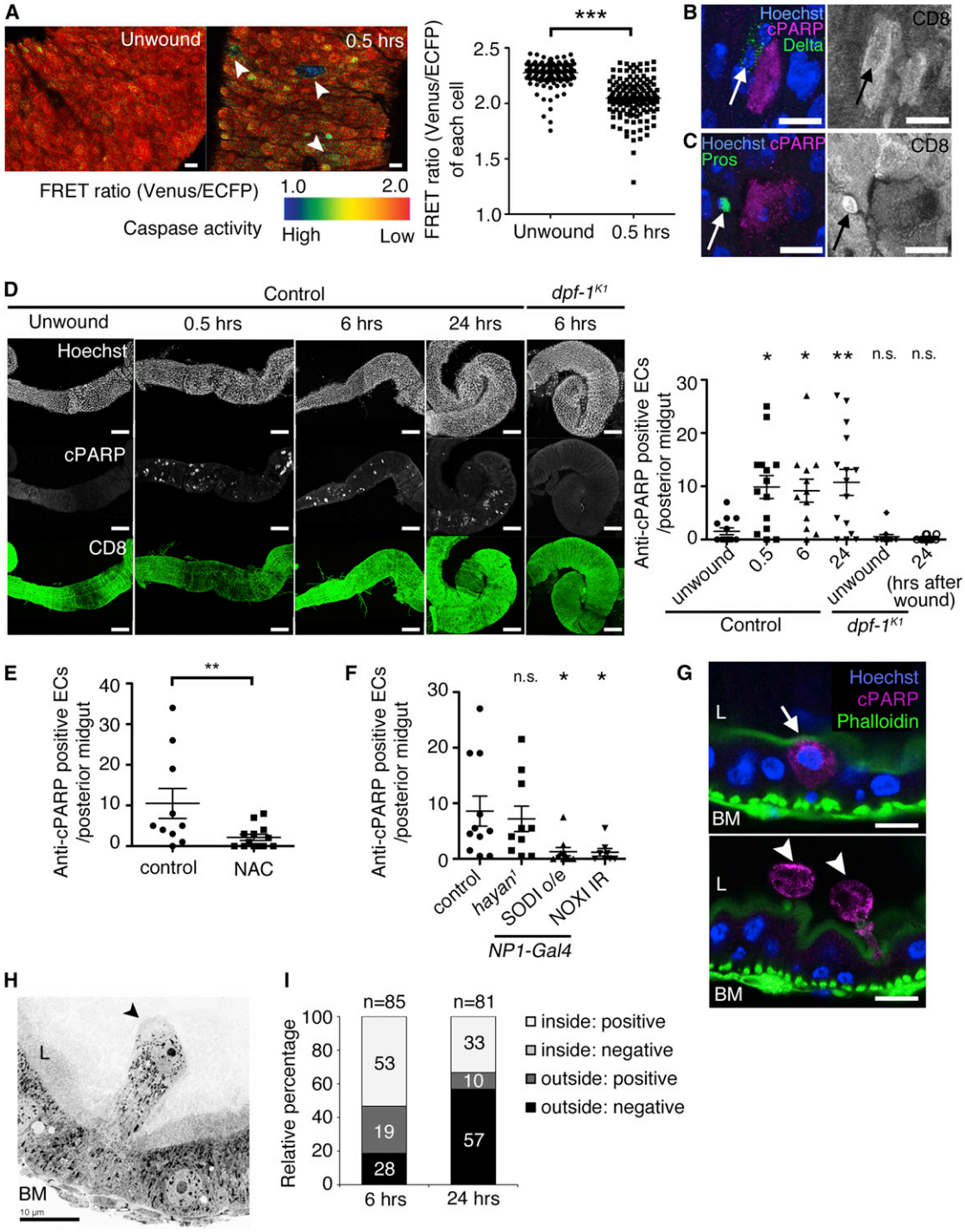

Figure 3. Caspase Is Activated in ECs after Wounding

(A) Left: Imaging analysis with SCAT3 in the gut of a control fly or a fly 30 min after wounding. High FRET (Venus/ECFP) ratio (red) indicates low caspase activity, and low FRET ratio (blue) indicates strong caspase activity. Some ECs (arrowheads) showed strong caspase activity. Right: The FRET ratio of individual cells is represented in the graph. Scale bars, 10 μm.

(B and C) Immunohistochemistry of the gut with CD8::PARP::Venus 24 hr after wounding. The cPARP-positive cells (magenta) were negative for (B, left) Delta (ISCs; green, arrow) and (C, left) Pros (EEs; green, arrow), although CD8::PARP::Venus was expressed in all of the cells (right panels of C and B for CD8 staining). Blue: Hoechst. Scale bars represent 10 μm.

(D) Immunohistochemistry of guts expressing CD8::PARP::Venus in the ECs. Left: top, white: Hoechst; middle, white: cPARP; bottom, green: CD8. Scale bars represent 100 μm. Right: Graph of the number of cPARP-positive cells in each gut after wounding.

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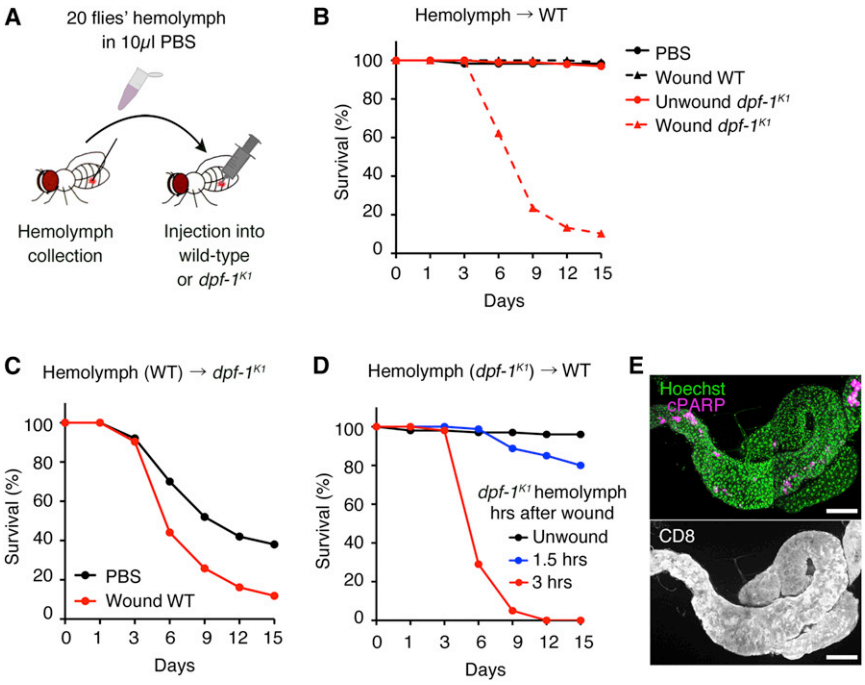

Figure 4. *dpf-1*^{K1} Hemolymph Contains a Lethal Factor after Wounding

(A) Experimental procedure for hemolymph injection. The hemolymph of 20 flies was collected into 10 μl of PBS, and 65 nL was injected into the abdomen of each recipient fly.

(B) Survival rate of WT flies after injection of PBS or hemolymph from wounded WT or unwounded or wounded *dpf-1*^{K1}flies.

(C) Survival rate of *dpf-1*^{K1} flies after injection of PBS or hemolymph from wounded WT flies.

(D) Survival rate of WT flies after injection of *dpf-1*^{K1} hemolymph collected at various time points after wounding. The lethal factor appeared within 3 hr after wounding.

(E) Caspase activity observed in a fly expressing CD8::PARP::Venus in the ECs 24 hr after injection of hemolymph from wounded *dpf-1*^{K1} flies. Green, Hoechst; magenta, cPARP; white, CD8. Scale bars represent 100 μm.

die from anoikis (Buchon et al., 2010), which is an apoptosis induced by the cell's detachment from the extracellular matrix.

We also classified the cPARP-positive cells in the gut 6 hr and 24 hr after wounding. In control flies, 53% of the cPARP-positive ECs were located in the gut cell layer 6 hr after wounding (Figure 3I). However, by 24 hr after wounding, 67% of the cPARP-positive ECs were located outside the gut cell layer at the luminal side (Figure 3I). The cPARP-positive ECs in the gut cell layer were positive for nuclear Hoechst 33342 staining (Figures 3G and 3I), but most of the ones outside of the layer were not (Figures 3G and 3I). These results suggest that the cPARP-positive ECs lost their nuclear DNA and, having been extruded from the gut cell layer, were undergoing apoptosis.

A Lethal Factor Exists in the Hemolymph of Wounded *dpf-1*^{K1}

We next examined why blocking the gut cell turnover by caspase inhibition resulted in fly death after wounding. Larval epithelial wounding induces humoral immune responses, such as antimicrobial peptide secretion, Pvf signal transduction, and JNK signal induction (Wu et al., 2009). In mammals, a systemic immune reaction is induced by damage-associated molecular pattern molecules (DAMPs), which are released from damaged cells that could not undergo apoptosis (Lotze et al., 2007; Scaffidi et al., 2002). We therefore hypothesized that a systemic factor contributes to the wound-induced lethal phenotype of *dpf-1*^{K1}.

To examine this possibility, we performed hemolymph transfer experiments. Fly hemolymph was collected with a microinjection needle into PBS by capillary action and injected into the abdomen of recipient flies (Figure 4A). The *dpf-1*^{K1} hemolymph taken 48 hr after wounding contained a lethal factor, but that taken from unwounded *dpf-1*^{K1} did not (Figure 4B). On the other hand, the hemolymph of pricked wild-type flies did not rescue the wound-induced lethal phenotype of *dpf-1*^{K1}, suggesting that the hemolymph of wounded wild-type animals does not contain a protective factor (Figure 4C). We also examined whether the hemolymph from pricked *dpf-1*^{K1} contained more bacteria than the other hemolymph samples. We plated the hemolymph samples used in this series of experiments on a lysogeny broth plate and confirmed that there was no bacterial growth on the plate of the hemolymph sample (data not shown). Further examination showed that the lethal factor was present in the wounded *dpf-1*^{K1} hemolymph 3 hr after wounding (Figure 4D). These results suggested that caspase activity in ECs suppresses the lethal factor from being released into the hemolymph after wounding.

Caspase activation in the gut of wounded wild-type flies occurred within 0.5 hr after wounding, which is earlier than the appearance of the lethal factor in the *dpf-1*^{K1} hemolymph (3 hr

(E) Number of cPARP-positive cells in each posterior midgut of flies 24 hr after wounding. NAC was administered in the food at 100 mM.

(F) Number of cPARP-positive cells in the posterior midgut of *Hayan* mutant flies or transgenic flies with *NP1-Gal4*-driven *SOD1* overexpression (o/e) or *NOX1* knockdown (IR) 24 hr after wounding.

(G) Confocal microscopy of the sagittal section of a control gut (24 hr after wounding). Magenta, arrow: cPARP-positive EC in the gut cell layer. Magenta, arrowheads: cPARP-positive ECs outside the cell layer. Blue, Hoechst; green, phalloidin. L, lumen. Scale bars represent 10 μm.

(H) EM of the WT gut 24 hr after wounding. Arrowhead: a dying EC. Scale bar represents 10 μm.

(I) Location and nuclear Hoechst staining of cPARP-positive ECs in a control fly 6 hr and 24 hr after wounding. Inside/outside of the gut cell layer: nuclear Hoechst positive/negative. The number of observed gut samples is shown on top of each column (n = 85, 81).

Error bars in all graphs indicate SEM. See also Figures S4 and S5.

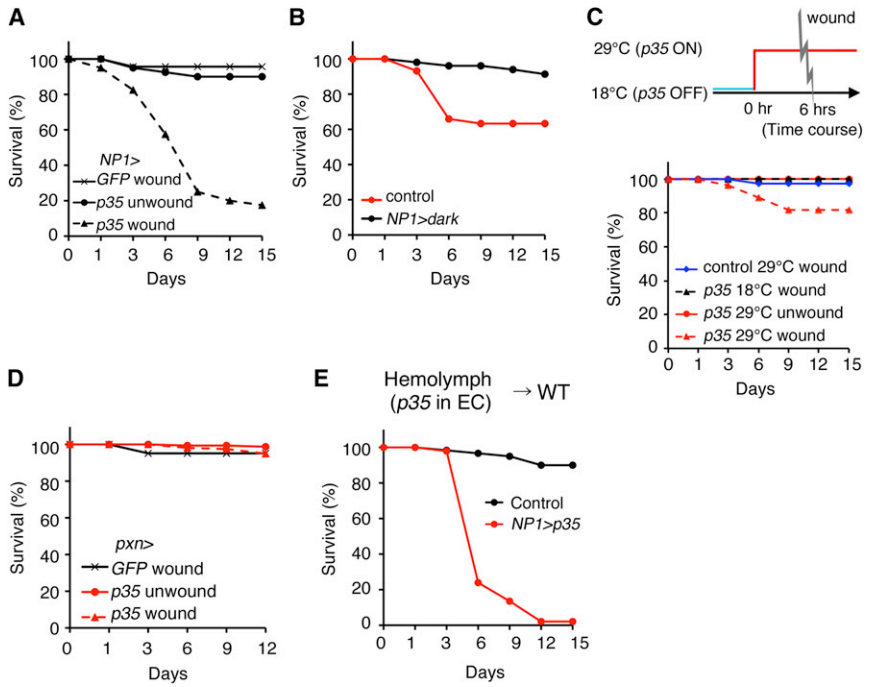

Figure 5. Caspase Activity in ECs Is Required for the Systemic Wound Response

(A–E) Survival rate of wounded flies.

(A) Flies expressing *GFP* or *p35* in the gut in ECs.

(B) The wound-induced lethal phenotype of *dpf-1*^{K1} (control) was rescued by the overexpression of *dark* in ECs (*NP1 > dark*).

(C) Experimental procedure for starting *p35* overexpression in adulthood before wounding (top). Beginning 6 hr before wounding, *p35* expression in ECs was induced by a temperature shift from 18°C to 29°C. Survival rate after wounding (bottom).

(D) Flies expressing *GFP* or *p35* in the hemocytes (blood cells) using the *Pxn-Gal4* driver.

(E) Survival rate of WT flies after injection of hemolymph from wounded control flies or wounded flies expressing *p35* in the ECs *(NP1 > p35)*. Hemolymph was taken 48 hr after wounding.

after wounding; Figure 4D). This result is consistent with the idea that a defect in caspase activation results in the induction of a lethal factor. cPARP-positive ECs were observed in the wild-type gut after the injection of hemolymph from wounded *dpf-1*^{K1}, indicating that the lethal factor does not function upstream of caspase to suppress its activation (Figure 4E).

Caspase Activity in ECs Is Required to Dampen the Lethal Factor

Because caspase activation was observed in the ECs of the gut but not in the other cell types examined (Figures 3B and 3C), we performed a wounding experiment on flies in which caspase activity was suppressed in the ECs. The wound was lethal in flies expressing the pan-caspase inhibitor *p35* in the ECs (Figure 5A). In addition, the wound-induced lethal phenotype of *dpf-1*^{K1} was rescued by overexpressing *dark* in the ECs (Figure 5B). These results suggested that caspase activity in the ECs, but not in other cell types, such as wounded epidermis cells and hemocytes, is required for fly survival after wounding.

To avoid the effects of caspase inhibition during development, we used a temperature-sensitive allele (*tub-Gal80*^{ts}) to start overexpressing *p35* in adulthood (Figure 5C). Wound-induced lethality was also observed in the flies expressing *p35* just before wounding (Figure 5C). We found a statistically meaningful difference between control flies cultured at 29°C and *p35*-expressing flies cultured at 29°C, which supports our idea that wound-induced caspases activation is critical for survival (Figure 5C). Caspase inhibition in the hemocytes did not result in lethality after wounding (Figure 5D), indicating that the caspase in hemocytes is not required for the protective response.

We next investigated whether the loss of caspase activity in the ECs was responsible for generating the lethal factor, by performing a hemolymph injection experiment. Hemolymph from wounded flies expressing *p35* in their ECs induced lethality in wild-type flies, indicating that the ECs' caspase activity was important in the wound response (Figure 5E). These results support the hypothesis that caspase activity in the ECs can decrease the amount of the lethal factor in the hemolymph upon wounding.

DISCUSSION

Our results show that adult gut cell turnover is critical for the maintenance of gut homeostasis and to avoid lethality from wounding. Although the gut structure was disorganized in *dpf-1*^{K1}, a mutant with a defect in gut cell turnover, this phenotype had little effect on fly survival under normal culture conditions. However, *dpf-1*^{K1} flies showed increased sensitivity to wounding, mediated by the inhibition of caspase in the ECs. We concluded that caspase activation in the ECs regulates gut cell turnover to suppress the release of a lethal factor into the hemolymph after wounding (Figure 6).

Caspase Activity in ECs Contributes to Homeostasis of the Midgut

Since caspase activity is suppressed throughout development and adulthood in *dpf-1*^{K1}, there are two possible mechanisms for the gut defects observed in this mutant: developmental impairment of the adult midgut, and disruption of midgut homeostasis caused by a cell-turnover defect. A previous report showed that caspase activity is not required for the replacement of larval midgut cells by adult midgut cells (Denton et al., 2009). However, caspase activation could be required for gut formation after metamorphosis. Indeed, in *dpf-1*^{K1} flies, the collagen IV-GFP signal was disrupted even without wounding. In the adult, signals from muscle cells, such as wingless and epidermal growth factor (EGF), are reported to mediate ISC proliferation (Buchon et al., 2010; Jiang and Edgar, 2009; Lin et al., 2008). The disorganized extracellular matrix and muscle layer in

dpf-1^{K1} may affect the release of mitogens from muscle during development. However, our results from the overexpression of *PTEN* or *p35* in adult flies (Figures 2E and 5C) indicate that caspase-driven intestinal renewal in adults is crucial for them to overcome wound-induced lethality, and inhibition of this renewal is sufficient to induce lethality after wounding. Thus, caspase's functions in adult gut cell renewal appear to be important for the systemic wound response.

As we observed using CD8::PARP::Venus, most of the cPARP-antibody-positive ECs appear to be excluded from the gut cell layer, suggesting that they are dying (Figures 3G–3I). We also observed a subtle decrease in the SCAT3 ratio, which can be used to quantify caspase activation more accurately than PARP, in many gut cells after cuticle wounding. It is also possible that the activated caspase in the gut has nonapoptotic roles. We previously showed that Wg signaling is regulated by a nonapoptotic caspase function via Shaggy during neural precursor development (Kanuka et al., 2005). Since Wg regulates ISC proliferation, a nonapoptotic function mediated by caspase activation may affect the cell turnover.

Cell-Cell Interaction May Regulate Epithelial Cell Cycling

In the *Drosophila* imaginal disc model, undead cells that receive apoptotic stimuli promote the expression of Dpp and Wg, which induce the proliferation of neighboring cells (Huh et al., 2004; Pérez-Garijo et al., 2004; Ryoo et al., 2004). In addition, damaged ECs secrete Upd3 to induce activation of the Wg, EGF receptor, and JAK/STAT signaling pathways in muscle cells, and their activation contributes to ISC proliferation and gut cell turnover (Buchon et al., 2010; Jiang et al., 2009). The dying ECs of the wounded fly may function similarly, i.e., by secreting factors to induce ISC proliferation, much as dying cells of hydra subjected to midgastric bisection secrete Wnt3 to promote head regeneration (Chera et al., 2009). Inhibition of caspase activation prevents both cell death and Wnt3 secretion in the hydra model. The inhibition of executioner caspase by *p35* also prevents Hh expression in photoreceptor neurons and compensatory proliferation (Fan and Bergmann, 2008). The inhibition of caspase activation in ECs might likewise prevent their expression of mitogens that promote ISC proliferation.

Although *upd3* is expressed in the gut after cuticle wounding (Figure S6), its expression level may be lower than that of *upd3* overexpressed by the GAL4-UAS system, which can induce massive ISC proliferation (Jiang et al., 2009). A previous report suggested that knocking down *upd3* does not affect ISC proliferation under normal culture conditions (Osman et al., 2012), which suggests that some other factor or factors, such as Wg, EGF, or ROS, are critical regulators for homeostatic ISC proliferation. These factors or their downstream signaling may be reduced in *dpf-1* mutants.

During *Drosophila* metamorphosis, the replacement of larval epithelial cells (LECs) is regulated through their competitive interaction with proliferating HBs (Nakajima et al., 2011; Ninov et al., 2007). The cell border between the HBs and LECs (called the replacement boundary) plays critical roles in coordinating the proliferation of HBs and death of LECs (Nakajima et al., 2011).

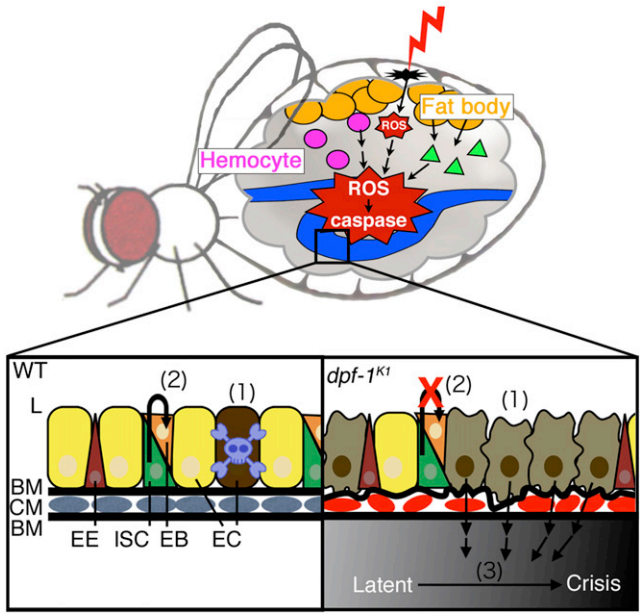

Figure 6. Model for the Caspase-Mediated Systemic Wound Response

Caspase activation is required to overcome wounding. ROS in ECs activate caspases. The fat body and hemocytes may also contribute to the systemic wound response, for example, by mediating signaling from the wound site to the gut or by generating cytokines as positive feedback. In wounded WT flies, caspase is activated to induce EC death (1) and ISC proliferation (2), resulting in gut epithelial turnover. In flies in which caspase is inhibited in ECs, gut homeostasis—i.e., EC death (1), gut cell turnover (2), gut cell repopulation, and gut structure—is impaired. Although these flies can survive under normal culture conditions, they are sensitive to wounding; the mechanism involves a lethal factor introduced into the hemolymph as a result of the wound. In other words, the wound changes the fly's condition from a latent phase to a crisis phase (3).

Similarly, because ISCs and ECs contact each other in the midgut, not only humoral factors but also the direct competitive interaction of proliferating ISCs and dying ECs might coordinate gut epithelial renewal. In this sense, a proliferation defect of ISCs could modulate the ECs' response to wounding, and might elicit the same situation as seen with EC death defects. This idea was supported by the wounding experiment using flies with an ISC proliferation defect (Figures 2D, 2E, and S5C). A cell-cell contact-mediated signaling pathway, such as the Hippo pathway, and lateral inhibition signaling through Notch and Delta are candidate mechanisms for such a direct interaction (de Navascués et al., 2012; Shaw et al., 2010).

Possible Role of the Gut as a Sensor for Danger Signals from Damaged Tissues

The gut is reactive to external factors such as diet and bacteria. To protect the gut epithelia from external factors, the insect intestinal lumen has a physical barrier called the peritrophic matrix, which is composed of chitin and glycoproteins (Kuraishi et al., 2011). The visceral muscles and basal lamina may act as a barrier for internal factors. However, they are not sufficient to prevent the access of signals from a wound site to the ECs, since

our present study indicates that ECs may produce ROS and induce caspase activation after epithelial injury. In mammals, JAK/STAT signaling pathway regulates NOX1 and NOX4 (Manea et al., 2010). Because Upd3 expression is upregulated in the epidermis and ECs after wounding in both wild-type and *dpf-1*^{K1} flies (Figure S6), it is possible that a JAK/STAT amplification loop between the wounded tissue, hemocytes, and fat body mediates signaling from the wound site to the gut (Pastor-Pareja et al., 2008). Although Hayan was not involved in the mechanism that induced caspase activation in the gut after wounding, it is still possible that the gut cell response is mediated through the nervous system, hemocytes, or ROS generated at the wound site. Furthermore, mammalian studies have suggested that damaged cells secrete diffusible molecules other than ROS, such as ATP, histones, and heat shock proteins (Chen and Nuñez, 2010). In addition to ROS, these molecules could mediate signals from the wound site to the gut in *Drosophila*.

Although further experiments are required to identify the toxic factor and the factor-producing tissue in the caspase-pathway-inhibited fly after wounding, we identified the gut as one of the organs responsible for overcoming tissue injury in *Drosophila*. The toxic factor could be a potent molecule, such as a protease or other enzyme or a hormone, since only a small amount of the hemolymph from wounded *dpf-1*^{K1} flies induced lethality in wild-type flies.

The stem cell system in recycling tissue seems to have a feature that differentiated cells are highly sensitive to stress and stem cells rapidly respond to the dying differentiated cells. The gut is one of the first organs to appear in multicellular organisms in evolution, and it is the largest organ in the body, extending through most of the body axis (Stainier, 2005). Thus, it can be speculated that the function of the gut epithelia as a sensitive sensor for external and internal danger signals is evolutionarily conserved. In this respect, caspase activation would be an effective way to produce factors for stem cell proliferation and cell replacement in the gut without excess activation of the inflammatory response.

EXPERIMENTAL PROCEDURES

Wounding Experiments

Canton S was used as wild-type. Unwounded flies were used as controls unless otherwise stated. Adult male flies were pricked with a microinjection needle on the abdomen 2–3 days after eclosion. The survivors were counted and transferred into a fresh culture vial 1 day after wounding and every 3 days for 15 days thereafter.

Hemolymph Injection

Hemolymph was collected with a microinjection needle from the thorax of 20 flies by capillary action and diluted into 10 μl of PBS on ice. After centrifugation, 65 nL of the hemolymph/PBS mixture was injected into the abdomen of the host fly.

Immunostaining

Fly midgut, epithelia, brain, and fat bodies were dissected in PBS and fixed for 15 min with 4% (vol/vol) paraformaldehyde/PEM (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$) buffer. Samples were washed three times for 10 min each with 0.02% (vol/vol) Triton X-100/PBS and incubated overnight at 4°C with the primary antibody and 5% donkey serum (vol/vol). The samples were washed three times for 10 min each with 0.02% (vol/vol) Triton X-100/PBS buffer and incubated for 2 hr at room temperature with the secondary antibody and 5% donkey serum (vol/vol). The samples were mounted with mounting buffer and analyzed by confocal microscopy (TCS SP5; Leica). X-gal staining was performed as described previously (Kamiya et al., 2011).

For further details regarding the materials and methods used in this work, see Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.022.

LICENSING INFORMATION

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