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The role of *Drosophila* antifungal immune response genes in intestinal homeostasis

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Abstract: The signaling pathways that control intestinal development, regeneration and disease show a high degree of conservation between *Drosophila* and mammals. The gut epithelia of *Drosophila* provide protection against invasion of microorganisms through production of reactive oxygen species (ROS) and antimicrobial peptides (AMPs). Although *Drosophila* gut immunity has been extensively studied, the specific responses to Gram-positive bacteria, fungi and toxic compounds are not fully understood. To identify the physiological role of genes involved in host defense we studied *Drosophila* mutants in antifungal genes identified previously and tested their survival upon feeding with various pathogens and toxic compounds. The results showed that several mutants displayed decreased viability compared with wild-type flies, and the lower survival rates were attributed to morphological change and excessive cell death in mutant guts. Thus, we identified several new *Drosophila* genes (*spen*, *jumeau*, *inv*, *DDB1* and *shg*) required for intestinal homeostasis or stress responses.

Keywords: *Drosophila*, intestinal homeostasis, antifungal genes, ingestion, stress response

1 Introduction

In addition to its role in nutrient absorption, the intestine is also a barrier between internal and external environment, which protects the host against invasion and systemic dissemination of both pathogenic and toxic components [1,2]. In *Drosophila*, the immune response of the gut mainly relies on the local release of antimicrobial peptides (AMPs) and the production of microbicidal reactive oxygen species (ROS) [3,4]. The local production of AMPs plays a critical role for the inducible defense mechanisms in the gut. In addition, the generation of ROS is also involved in host defense against microbes within the *Drosophila* gut [5]. The ROS can damage DNA, RNA and protein, and promote the oxidative degradation of lipids in the cell membranes of a microbe. Furthermore, ROS also stimulates intestinal stem cell (ISC) proliferation. The balance between cell damage and epithelial repair maintains *Drosophila* gut homeostasis after pathogen infection. The epithelial renewal is required for maintaining gut homeostasis and survival, and plays an essential role in barrier against tissue damage caused by the ingestion of pathogens and toxic compounds [6]. However, excessive ROS can also damage the host intestinal epithelium, therefore the balance between production and removal of ROS is essential for the health of the host [7]. Besides, *Drosophila* midgut can rapidly regenerate after subjected to damage caused by oxidative stress, and several conserved signaling pathways including JAK-STAT pathway, Ras/MAPK signaling and p38b/MK2 signaling participate in the renewal and proliferation of intestinal epithelial cell [8-10].

To date, most analyses on the intestinal immune response have focused on Gram-negative bacterial species, such as *Erwinia carotovora 15* (*Ecc-15*), *Pseudomonas entomophila* (*P. entomophila*) and *Serratia marcescens* (*S. marcescens*) [11-13]. However, specific responses to Gram-positive bacteria, fungi and some toxic compounds are not fully understood. In our previous studies, fifteen genes have been identified to be induced in SL2 cells after treatment with LPS/PGN or curdlan using *Drosophila* cDNA microarrays [14]. Moreover, these genes were also shown to be required in the antifungal systemic immune response by examining the survival and AMP genes expression in individual mutations following *B. bassiana* infection [15]. Some of these genes are involved in diverse

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aspects of cellular and humoral immune response; for example. *spen, coro, shg, loco, Rab6* and *jhl-21* are required for phagocytosis of invading microbes. In addition, *spen, Trx-2, coro* and *CG6181* appear to be required for the expression of antimicrobial peptide genes upon fungal infection [15]. To further evaluate the role of the antifungal genes in *Drosophila* physiology, we examined survival rates in relevant mutant flies upon feeding with various kinds of pathogens and toxic compounds. The results indicated some of the mutants showed lower survival rates than wild-type flies, and the reduced survival rates were attributed to morphological change and increased epithelia cell death in mutant guts. These results may lay the foundation for defining a new class of genes that are involved in *Drosophila* intestinal homeostasis.

2 Materials and Methods

2.1 *Drosophila* strains

Drosophila melanogaster strains were cultured on a standard cornmeal yeast medium at 25°C and 60% humidity. *W1118* flies were used as wild-type control. Mutant flies containing a *P*-element at the translated/ untranslated region of the candidate genes (Table S1) were purchased from GenExel Stock Center (Daejeon Korea).

2.2 Survival experiments

B. bassiana and *M. luteus* have been previously described [15]. Survival and feeding experiments were performed following the procedure of Ha *et al.* [7]. Briefly, three- to five-day-old adult flies (15 female and 15 male) were dehydrated for 2 h without food, and then transferred into a vial containing five filter papers hydrated with 5% (w/v) sucrose solution contaminated with concentrated microbe solution (OD = 2 for spores of *B. bassiana*; OD = 150 for *M. luteus*) or hydrogen peroxide (1%, v/v; Sigma). The filter papers were changed every day; control flies were fed a solution containing only 5% sucrose solution. The SDS (0.5%, w/v) or NaCl (0.4M) was mixed with standard cornmeal yeast medium, and the vials were changed every other day. The flies fed standard cornmeal yeast medium only were used as a control of the both toxic compounds. The experiments were repeated independently at least three times.

2.3 Imaging and 7-AAD staining

Approximately 8-10 adult females were used for gut dissection. The entire gastrointestinal tracts were dissected in phosphate-buffered saline (PBS) and immediately observed using a microscope. For 7-AAD staining, dissected guts of various genotypes were stained with 7-AAD (5 μg/ml in PBS; Invitrogen, Carlsbad, CA) for 30 min in a humidified chamber, and then washed in PBS. Guts were fixed with 4% formaldehyde in PBS for 30 min and analyzed using an Axioskop 2 plus microscope (Zeiss). The experiments were repeated at least three times independently.

2.4 Quantitative real-time PCR

Total RNA was isolated from 20 dissected guts (without Malpighian tubules) with TRIzol (Invitrogen, Carlsbad, CA), and used for cDNA synthesis with M-MLV Reverse Transcriptase (Promega). The real-time PCR was performed using SYBR**R** Select Master Mix (Applied Biosystems) on an ABI PRISM 7500 system (Applied Biosystems). The results were normalized to the level of *RpL32* mRNA in each sample. The primer sequences used were as follows: *upd3* forward, 5'-CACGTACATGCGCAACATC-3', *upd3* reverse, 5'-TCCACGCTGCAGAGCAC-3'; *dro3* forward, 5'-CTAATGGAGGCCAACACTGTT-3', *dro3* reverse, 5'-TCCACTGACATGTCCCTCCT-3'; *RpL32* forward, 5'-AGTCGGATCGATATGCTAAGCTGT-3', *RpL32* reverse, 5'-TAACCGATGTTGGGCATCAGATACT-3'.

2.5 Statistical analysis

Statistical analysis was performed using a two-tailed unpaired Student's t-test using Prism software (GraphPad). A $^{**}p$ value < 0.01 was considered statistically significant and $^{***}p$ value < 0.001 statistically highly significant.

3 Results

3.1 Analysis of gut-associated immune defective mutants flies

In previous study we have identified 15 genes involved in the antifungal immune response, and the results showed that complex immune reactions are required to defend against *Beauveria bassiana* (*B. bassiana*) infection in *Drosophila* [15]. To discover the role of these genes in intestinal homeostasis, the mutant flies were used in oral infection with *B. bassiana*. Adult flies from each of the

mutants were fed with sucrose solution containing spores of *B. bassiana*, an entomopathogenic fungus that can infect many insect species by penetrating their cuticles [16]. In the control experiment, all tested flies displayed high survival rates after feeding with sucrose solution (Fig. 1A). After oral infection with spores of *B. bassiana*, only *spen* mutants showed a minor decrease in survival and other mutants showed no significant defect in survival rate (Fig. 1B; Table S2). This result demonstrated that these antifungal genes do not contribute to the protection against ingested *B. bassiana*. To identify genes involved in the intestinal homeostasis, we next employed the same experiment using Gram-positive bacteria (*Micrococcus. luteus*) infection. All tested flies showed high survival rates except the *jumeau* and *loco* mutants. The *jumeau* displayed 32.2% and 78.9% mortality after 3 and 6 days respectively, while the *loco* mutant showed minor defect in the survival rate after 6 days (50%) (Fig.1C). When the mutant flies were fed with Gram-negative bacteria *Ecc-15*, all mutants showed no significant defect in survival rates (data not shown). Thus, we found that the mutations in *jumeau* and *loco* genes were associated with lower survival rates after feeding with *M. luteus*.

3.2 Analysis of intestinal stress response genes using mutant flies

Some toxic compounds can affect gut homeostasis by inducing damage and apoptosis of epithelial cells [6,17]. To identify genes involved in gut homeostasis, adult flies were exposed to various compounds, including H_2O_2, SDS and NaCl. The mutants of *spen*, *jumeau*, *inv* and *DDB1* showed significant lethality with survival rates of less than 20% after feeding with H_2O_2 (Fig. 1D, Table S2). Furthermore, we observed the increased lethality in *spen*, *shg* and *jumeau* mutant compared with wild-type flies upon SDS ingestion, and only *shg* mutants displayed osmotic stress (0.4 M NaCl). However, in control experiment, all mutants grew well in standard cornmeal medium (Fig. 2, Table S2).

3.3 Reduced survival rates due to the morphological change and increased cell death in the digestive tract

To determine whether the morphological change and cell death contribute to the high susceptibility of mutant flies to stressor ingestion, we observed the histological changes

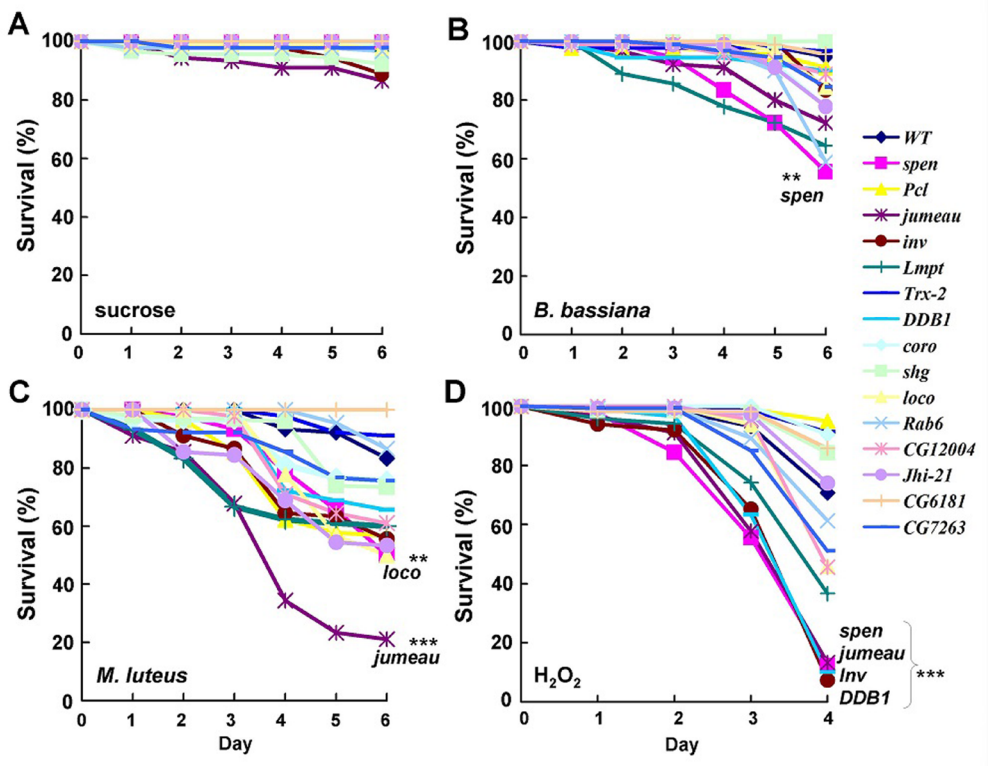

Fig. 1. Survival rates of wild-type and mutant flies following oral infection with pathogen and hydrogen peroxide. The wild-type and mutant flies were fed with spores of *B. bassiana* (B), *M. luteus* (C) and hydrogen peroxide (H_2O_2) for 4-6 days (D). The flies treated with 5% sucrose alone were used as the control (A). The color code of each line is the same as in the right side of the figure. Kinetics of survival rate from at least three independent experiments is shown. *p*-values were calculated by Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

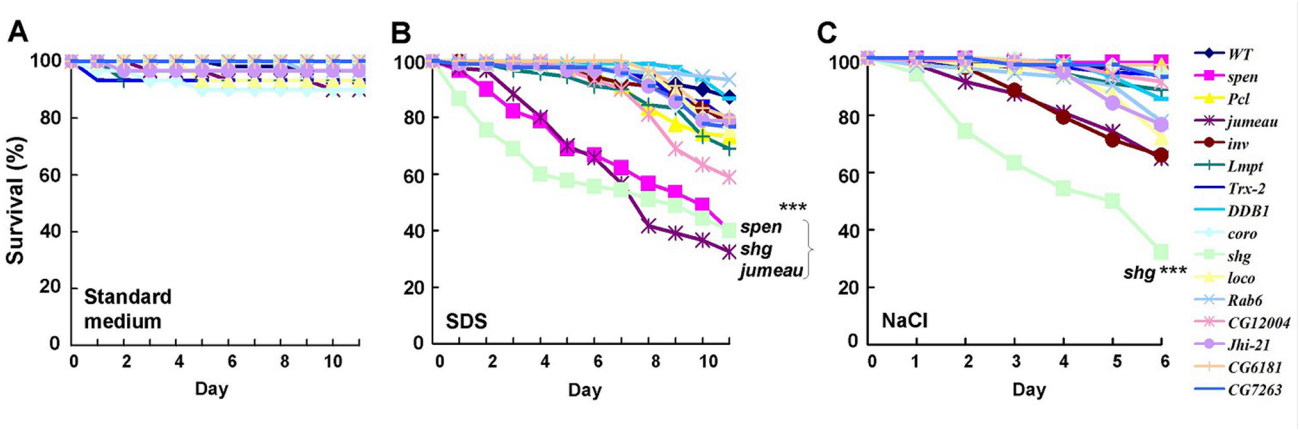

Fig. 2. Survival rates of wild-type and mutant flies following oral infection with toxic compounds. The mutant flies were fed with SDS (0.5%) (B) and NaCl (0.4 M) (C) for 6-11days. The flies fed with standard cornmeal medium were used as the control (A). The color code of each line is the same as in the right side of the figure. Kinetics of survival rate from at least three independent experiments is shown. *p*-values were calculated by Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

in the digestive tracts of *spen*, *jumeau* and *shg* mutants which showed low survival rates upon SDS ingestion. After feeding with 0.5% SDS for 96 h, the crops appeared to completely atrophy in *spen*, *jumeau* and *shg* mutants. Compared with the midguts of either challenged wild-type or unchallenged mutant flies, the epithelial lining became thinner in the midgut of *spen*, *jumeau* and *shg* mutant treated with SDS. Besides, large numbers of melanotic masses were observed in the hindgut of *spen*, *jumeau* and *shg* mutant flies.

Furthermore, we speculated that *spen*, *jumeau* and *shg* genes may be specifically required to protect the midgut epithelium. The cell death in the intestinal epithelium was determined by 7-AAD staining. The mutants fed with SDS for 144 h showed a dramatic increase in the number of dead cells in *spen*, *jumeau* and *shg* mutant compared with unchallenged mutant or challenged wild-type guts. Besides, few dead cells were observed in unchallenged *jumeau* mutant guts (Fig. 3C).

3.4 Analysis of *dro3* and *upd3* levels in mutant guts after SDS ingestion

A previous study suggested that the JAK-STAT pathway participates in the stress response of the intestine as expression of *upd3* (JAK-STAT pathway) was induced after SDS ingestion [2]. Additionally, *dro3* (*Drosomycin*-like peptide) appears specific to the gut immune response, and is regulated by the JAK-STAT pathway [2]. To examine whether *spen*, *jumeau* and *shg* are involved in the activation of the JAK-STAT pathway, we analyzed the expression levels of *dro3* and *upd3* in adult mutant guts by real-time PCR following feeding with 1% SDS for 16 h (Fig. 4). The *dro3* levels were significantly reduced in unchallenged (4- and 5.9-fold) and challenged (13- and 8.7-fold) *spen* and *jumeau* mutant, however, the *upd3* levels showed no obvious difference between mutant and wild-type guts. In addition, the expression levels of *dro3* and *upd3* were not significantly different in unchallenged and challenged *shg* mutant guts compared with wild-type guts (Fig. 4). This result indicates that the *spen* and *jumuea* may be involved in JAK-STAT pathway in intestinal homeostasis. However, the regulatory mechanisms of the two genes in intestinal immune response remain to be investigated.

4 Discussion

The research of systemic immune response has been extensive in *Drosophila* and these studies profoundly impacted our understanding of insect defense and homeostatic mechanisms. The gut homeostasis is ensured by the complex interaction between the intestinal epithelium and the gut microbes. Furthermore, studies in the *Drosophila* model provided new insights into intestinal maintenance and homeostasis. In previous studies we have identified fifteen genes that are specifically required for survival following *B. bassiana* septic infection. To evaluate the role of these genes in *Drosophila* physiology, we analyzed the effect of fifteen mutants on intestinal homeostasis and organism survival after ingestion of some pathogens and chemicals. Unlike the systemic immune response, only a few of these mutants (*jumeau*, *spen*, *shg*, *inv* and *DDB1*) showed immune defects and were susceptible to pathogen or stressor ingestion, yet most of the other mutants showed no obvious defect in survival (Table S2, Table S3).

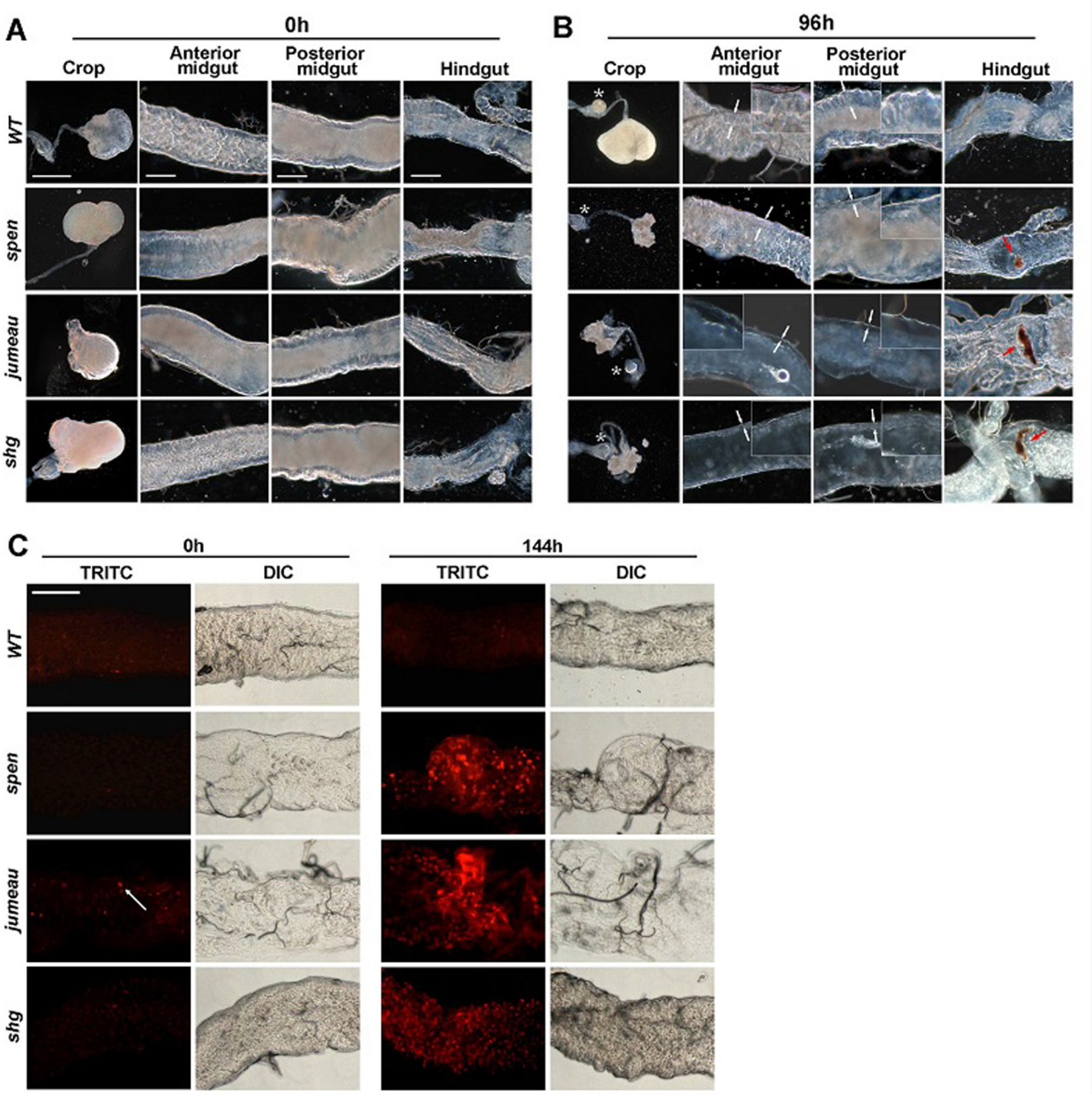

Fig. 3. The SDS induced significant morphological change and epithelial cell death in the digestive tract of mutant flies. (A, B) Brightfield images of the crop, midgut and hindgut of an adult female (data from 8-10 adults) after ingestion of 5% sucrose (A) or 0.5% SDS (containing 5% sucrose) (B) for 96 h. The squared box represents the enlarged image. Images are representative of at least three independent experiments. Asterisk: proventriculus; white arrow: intestinal epithelial cell layer; red arrow: melanotic mass. Scale bar: crop, 500 μm; gut, 100 μm. (C) The wild-type and mutant females (from 8-10 adults) were fed with 0.5% SDS for 144 h. The dead cells of anterior midgut were detected with 7-AAD staining. 0 h: without infection; 144 h: infection for 144 h. TRITC (7-AAD): dead cells (red); DIC: Nomarski images. Scale bar: 100 μm. Images are representative of at least three independent experiments.

Jumeau is a transcription factor expressed in embryonic central nervous systems (CNS) and can regulate development, nuclear morphology and function, and chromatin organization of *Drosophila* [18]. *Jumeau* mutants had a higher mortality after *M. luteus*, H_2O_2 and SDS ingestion, but there was no obvious effect on viability after feeding with *B. bassiana* and NaCl. We also observed a few dead cells in untreated *jumeau* mutant guts, however, the function of *jumeau* in gut immune responses need to be investigated.

The gene *spen* plays essential roles in the chromatin modification needed for hemocyte development [15,19]. Previous studies suggested that *spen* mutants were highly sensitive to *B. bassiana* and *M. luteus* septic infection

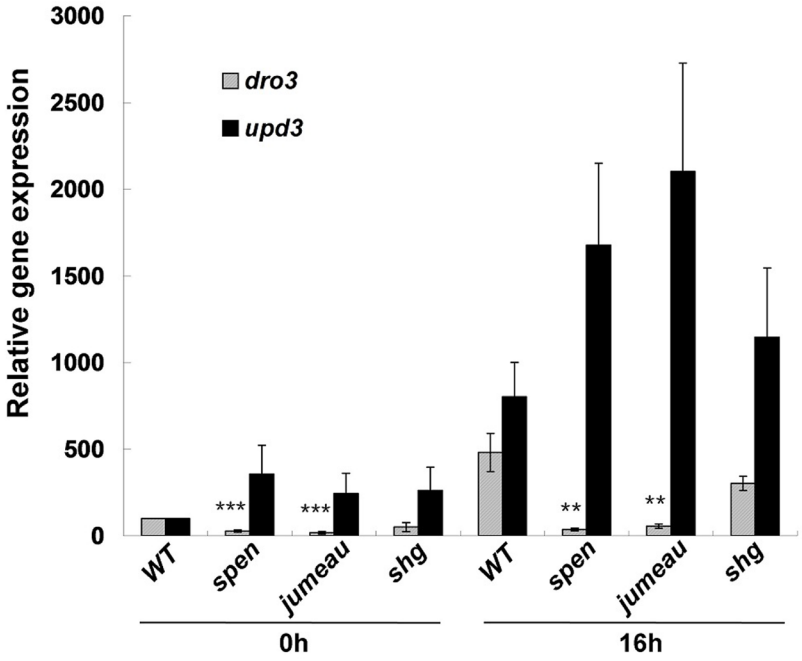

Fig. 4. Expression levels of dro3 **and** upd3 **in wild-type and mutant guts after SDS ingestion.** Real-time PCR analysis of *dro3* and *upd3* levels from gut extracts. 0 h: unchallenged flies; 16 h: ingestion of 5% sucrose containing SDS (final 1%) for 16 h. The number of transcripts in each sample was normalized to *RpL32* levels. The mean and standard deviation of three independent experiments are shown. $^{**}p < 0.01$, $^{***}p < 0.001$.

[15], however, our results showed only a minor decrease in survival rate after feeding with *B. bassiana* (Table S2, Table S3). Unexpectedly, *spen* caused severe increase of mortality after H_2O_2 and SDS ingestion.

Shg is a *Drosophila* Cadherin and is required for cell motility, adhesion and phagocytose fungi and bacteria [20-22]. Shg mutant was specifically sensitive to SDS and osmotic stress (0.4 M NaCl), but showed a high survival rate equivalent to that of wild-type flies after fungal and Gram-positive bacterial ingestion.

Among the fifteen genes, *inv* and *DDB1* are specifically required for defense against H_2O_2 ingestion. DDB1 is involved in the recognition of damaged DNA in dying cells or invading pathogens and crystal cells development [15,23]. Inv is a transcription factor that is involved in hindgut development via Delta-Notch signaling [24]. However, the way in which inv and DDB1 regulate the oxidative stress signaling in the gut remains to be determined. The ingestion of toxic compounds such as SDS or dextran sulfate sodium (DSS) was demonstrated to induce epithelium damage [2,25]. After SDS ingestion, atrophic crops, melanotic masses, thin epithelial layers and increased dead cells appeared in the guts of three mutants (*spen*, *jumeau* and *shg*) (Fig. 3). Therefore, the gut homeostatic balance between cell damage induced by pathogens or stressors and epithelial renewal may be disrupted in mutant flies. Besides, Sibley *et al*. observed that destruction of the epithelial layer and musculature of the crop likely impairs normal digestive function [20]. Therefore, morphological change and increased cell death in three mutant flies may contribute to the lower survival rate after SDS ingestion.

In conclusion, our study has determined several genes involved in the intestinal homeostasis and its ability to serve as a barrier against different stressors. The results have established a basis for further analyses of the intestinal homeostasis and the gut immune response in *Drosophila*.

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Conflict of interest: Dr. Jin has nothing to disclose.

References

[1] Sansonetti P. J., War and peace at mucosal surfaces, Nat. Rev. Immunol., 2004, 4, 953-964.

[2] Buchon N., Broderick N. A., Poidevin M., Pradervand S., Lemaitre B., *Drosophila* Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation, Cell Host Microbe, 2009, 5, 200-211.

[3] Ha E. M., Oh C. T., Bae Y. S., Lee W. J., A direct role for dual oxidase in *Drosophila* gut immunity, Science, 2005, 310, 847-850.

[4] Ferrandon D., Imler J. L., Hetru C., Hoffmann J. A., The *Drosophila* systemic immune response: Sensing and signalling during bacterial and fungal infections, Nat. Rev. Immunol., 2007, 7, 862-874.

[5] Ryu J. H., Ha E. M., Oh C. T., Seol J. H., Brey P. T., Jin I., et al., An essential complementary role of NF-kappaB pathway to microbicidal oxidants in *Drosophila* gut immunity, EMBO J., 2006, 25, 3693-3701.

[6] Bouchon N., Broderick N. A., Chakrabatti S., Lemaitre B., Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*, Genes Dev, 2009, 23, 2333-2344.

[7] Ha E. M., Oh C. T., Ryu J. H., Bae Y. S., Kang S. W., Jang, I. H., et al., An antioxidant system required for host protection against gut infection in *Drosophila*, Dev. Cell, 2005, 8, 125-132.

[8] Jiang H., Patel P. H., Kohlmaier A., Grenley M. O., McEwen D. G., Edgar B. A., Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut, Cell, 2009, 137, 1343-1355.

[9] Seisenbacher G., Hafen E., Stocker H., MK2-dependent p38b signalling protects Drosophila hindgut enterocytes against JNK-induced apoptosis under chronic stress, PLoS Genet., 2011, 7, e1002168.

[10] Ragab A., Buechling T., Gesellchen V., Spirohn K., Boettcher A. L., Boutros M., Drosophila Ras/MAPK signalling regulates innate immune responses in immune and intestinal stem cells, EMBO J., 2011, 30, 1123-1136.

[11] Nehme N. T., Liégeois S., Kele B., Giammarinaro P., Pradel E., Hoffmann J. A., et al., A model of bacterial intestinal infections in *Drosophila* melanogaster, PLoS Pathog., 2007, 3, e173.

[12] Buchon N., Osman D., David F. P., Fan H. Y., Boquete J. P., Deplancke B. et al., Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*, Cell Rep., 2013, 3, 1725-1738.

[13] Basset A., Khush R. S., Braun A., Gardan L., Boccard F., Hoffmann J. A., et al., The phytopathogenic bacteria *Erwinia carotovora* infects *Drosophila* and activates an immune response, Proc. Natl Acad. Sci. USA, 2000, 97, 3376-3381.

[14] Jin L.H., Choi J.K., Cho H., Shim J., Kim Y.J., Microarray analysis of the gene expression profiles of SL2 cells stimulated by LPS/PGN and Curdlan, Mol. Cells, 2008, 25, 553–558.

[15] Jin L. H., Shim J., Yoon J. S., Kim B., Kim J., Kim-Ha J., et al., Identification and functional analysis of antifungal immune response genes in *Drosophila*, PLoS Pathog., 2008, 4, e1000168.

[16] Lemaitre B., Hoffmann J. A., The host defense of *Drosophila* melanogaster, Annu. Rev. Immunol., 2007, 25, 697-743.

[17] Buchon N., Broderick N. A., Chakrabarti S., Lemaitre B., Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*, Genes Dev., 2009, 23, 2333-2344.

[18] Hofmann A., Brünner M., Schwendemann A., Strödicke M., Karberg S., Klebes A., et al., The winged-helix transcription factor JUMU regulates development, nucleolus morphology and function, and chromatin organization of *Drosophila melanogaster*, Chromosome Res., 2010, 18, 307-324.

[19] Shi Y., Downes M., Xie W., Kao H. Y., Ordentlich P., Tsai C. C., et al., Sharp, an inducible cofactor that integrates nuclear receptor repression and activation, Genes Dev., 2001, 15, 1140-1151.

[20] Sibley C. D., Duan K., Fischer C., Parkins M. D., Storey D. G., Rabin H. R., et al., Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections, PLoS Pathog., 2008, 4, e1000184.

[21] Tepass U., Gruszynski-DeFeo E., Haag T. A., Omatyar L., Török T., Hartenstein V., Shotgun encodes *Drosophila* E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia, Genes Dev., 1996, 10, 672-685.

[22] Tanaka-Matakatsu M., Uemura T., Oda H., Takeichi M., Hayashi S., Cadherin-mediated cell adhesion and cell motility in *Drosophila* trachea regulated by the transcription factor Escargot, Development, 1996, 122, 3697-3705.

[23] Takata K., Shimanouchi K., Yamaguchi M., Murakami S., Ishika wa G., Takeuchi R., et al., Damaged DNA binding protein 1 in *Dr osophila* defense reactions,
Biochem. Biophys. Res. Commun., 2004, 323, 1024–1031.

[24] Iwaki D. D., Lengyel J. A., A Delta-Notch signaling border regulated by Engrailed/Invected repression specifies boundary cells in the *Drosophila* hindgut, Mech. Dev., 2002, 114, 71-84.

[25] Amcheslavsky A., Jiang J., Ip Y. T., Tissue damage-induced intestinal stem cell division in *Drosophila*, Cell Stem Cell, 2009, 4, 49-61.

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