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An essential complementary role of NF-κB pathway to microbicidal oxidants in *Drosophila* gut immunity

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In the *Drosophila* gut, reactive oxygen species (ROS)-dependent immunity is critical to host survival. This is in contrast to the NF-κB pathway whose physiological function in the microbe-laden epithelia has yet to be convincingly demonstrated despite playing a critical role during systemic infections. We used a novel *in vivo* approach to reveal the physiological role of gut NF-κB/antimicrobial peptide (AMP) system, which has been 'masked' in the presence of the dominant intestinal ROS-dependent immunity. When fed with ROS-resistant microbes, NF-κB pathway mutant flies, but not wild-type flies, become highly susceptible to gut infection. This high lethality can be significantly reduced by either re-introducing Relish expression to Relish mutants or by constitutively expressing a single AMP to the NF-κB pathway mutants in the intestine. These results imply that the local 'NF-κB/AMP' system acts as an essential 'fail-safe' system, complementary to the ROS-dependent gut immunity, during gut infection with ROS-resistant pathogens. This system provides the *Drosophila* gut immunity the versatility necessary to manage sporadic invasion of virulent pathogens that somehow counteract or evade the ROS-dependent immunity.

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Introduction

Gastrointestinal epithelia face an exceptional challenge among various organ tissues in that they are in constant contact with a countless number of microbes (Macpherson and Harris, 2004; Sansonetti, 2004; Macdonald and Monteleone, 2005). Therefore, this microbial-laden mucosal tissue must be armed with an efficient innate microbial control system. (Ganz, 2003; Bevins, 2004; Lehrer, 2004). In *Drosophila* gut, intestinal redox homeostasis, via the infection-induced *de novo* generation of oxygen-dependent innate immune effectors such as reactive oxygen species (ROS) by dual oxidase (Duox) and their elimination by immune-regulated catalase, is finely regulated to mediate pathogen–host interaction (Ha *et al*, 2005a, b). The function of this immune system is critical in the host survival during natural gut infections resulting for example from the ingestion of microbe-contaminated foods (Ha *et al*, 2005a, b). Natural gut infections can also trigger the *immune deficiency* (IMD)/NF-κB pathway in the intestine, which results in the *de novo* synthesis of innate immune effectors including antimicrobial peptides (AMPs) via the activation of p105-like NF-κB, Relish (Ferrandon *et al*, 1998; Tzou *et al*, 2000; Onfelt Tingvall *et al*, 2001). Despite the central role of the NF-κB/AMP pathway in host survival during the systemic immune response, which follows microbial infection in the hemocoel (Silverman and Maniatis, 2001; Boutros *et al*, 2002; Hoffmann and Reichhart, 2002; Hultmark, 2003; Brennan and Anderson, 2004; Lemaitre, 2004), its exact physiological function in intestinal innate immunity has not yet been convincingly demonstrated at the organism level. This is probably attributed to the fact that other effective defense systems such as ROS-dependent innate immunity are also operating in the gut and effectively controlling the majority of infections. Thus, at least under infectious conditions with a fairly wide spectrum of microbes, the epithelial NF-κB/AMP pathway appears to be less essential for host survival, as all known NF-κB mutant flies are totally resistant to natural gut infection (Ha *et al*, 2005a, b). Nevertheless, given that AMPs have been demonstrated *in vitro* to be capable of killing a wide variety of microbes (Hertu *et al*, 1998), we hypothesized that epithelial AMPs operating via the NF-κB pathway may constitute an essential antimicrobial defense within the gastrointestinal tract. We further hypothesized that this defense system may possibly exhibit a complementary and/or synergistic action in combination with the other efficient immune effectors, ROS, in *Drosophila* gut immunity. Clear *in vivo* data supporting or undermining this hypothesis is lacking at present, perhaps mainly owing to the absence of a suitable experimental model. In the present study, we show that intestinal NF-κB/AMP-dependent innate immunity becomes crucial to host survival when the host encounters pathogenic microbes that somehow escape ROS-dependent innate immunity. These results imply that the epithelia of *Drosophila* developed two evolutionally distinct innate immune effectors, ROS and AMPs. Such 'dual-effector' system in the *Drosophila* gastrointestinal epithelium makes it difficult for

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pathogens to completely resist or circumvent the host immunity thus insuring host survival.

Results

IMD/NF-κB pathway is required for host protection against gut infection by ROS-resistant microbes, but not by normal ROS-sensitive microbes

Recently, we have demonstrated that the ROS-dependent immune system, rather than the NF-κB-dependent innate immune system, is crucial to the survival of the host during the majority of host–microbe interactions in the gastrointestinal tract of *Drosophila* (Ha *et al*, 2005a, b). These observations also imply that, during continuous gut–microbe interactions, one of the principal tactics of microbes may involve the evasion of or resistance to the host's ROS system, thereby securing a foothold for proliferation within the host. To investigate whether ROS resistance is the major virulent mechanism of microbes, it would be ideal to establish the natural infection conditions with a microbe that exhibits a marked resistance to ROS. However, at present, no orally transmitted and ROS-resistant natural pathogens for *Drosophila* are known. Therefore, we used the KNU5377 yeast strain, isolated from a natural environment and highly resistant to various types of exogenous stresses (Kwak *et al*, 2003). In the ROS resistance test using various concentrations of hydrogen peroxide, the KNU5377 showed a much higher survival rate when compared to a standard yeast strain (W303) (Figure 1A). We then performed gut infection using a standard yeast strain (W303) and a ROS-resistant strain (KNU5377). Contrary to our expectation, the flies were totally resistant to ROS-resistant KNU5377 infection, and no significant difference in host mortality was observed between ROS-sensitive W303 infection and ROS-resistant KNU5377 infection (Figure 1B). This result suggests that another form of gut immune system may be also operating as a complementary system to ROS-dependent immunity for the efficient control of ROS-resistant microbes.

As natural infection is also known to activate local intestinal NF-κB pathway, we hypothesized that *Drosophila* relies on the intestinal NF-κB-dependent innate immunity as the second line of defense for the efficient host protection against ROS-resistant microbes. If this were the case, ROS-resistant microbial strains should prove to be more pathogenic to NF-κB pathway mutant flies than to normal flies as host survival would be largely dependent on the intestinal NF-κB pathway-dependent innate immunity. In an attempt to assess this hypothesis, we fed various IMD/NF-κB pathway mutant flies (p105-like NF-κB mutant (*Relish*^{E20}), caspase mutant (*Dredd*^{B118}) and *Drosophila* IκB kinase γ mutant (*key*^{1})) on either the KNU5377 strain or the W303 strain. Consistent with our hypothesis, high mortality levels were observed in these NF-κB pathway mutant flies only when they fed on KNU5377 strain (Figure 1C). No significant mortality was observed in the NF-κB pathway mutant flies fed on the W303 strain (Figure 1C). Interestingly, enhanced levels of KNU5377-induced mortality were observed only in the IMD/NF-κB pathway mutant flies (*Dredd*^{B118}, *key*^{1} and *Relish*^{E20}) but not in the Toll/NF-κB pathway mutant flies (*spz*^{rm7} and *Dif*^{1}) (Figure 1C). To rule out partially redundant function of three NF-κB molecules (Dif and Dorsal for Toll pathway and Relish for IMD pathway) in the gut immunity,

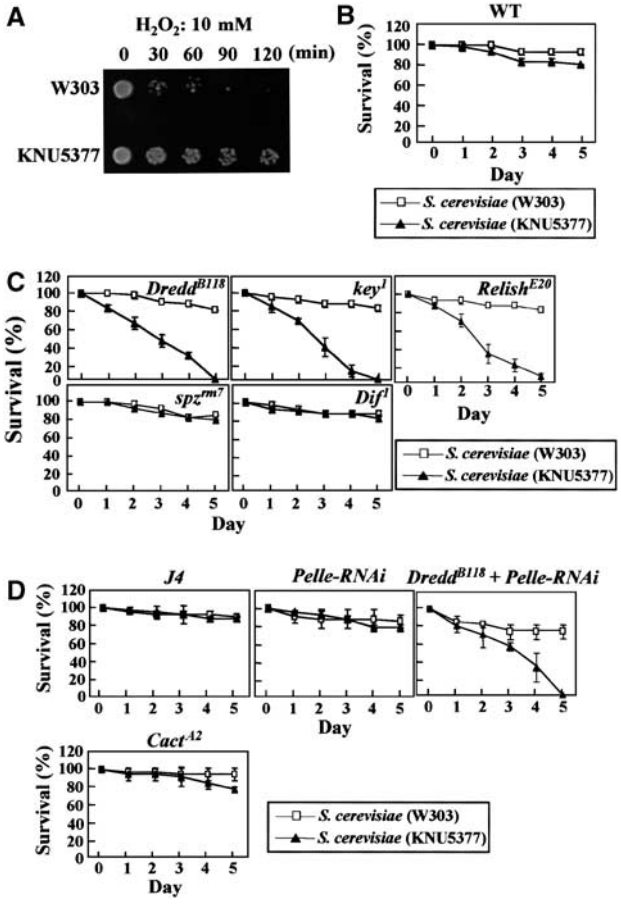

Figure 1 IMD/NF-κB-dependent innate immunity is indispensable for host protection from ROS-resistant pathogen. (**A**) KNU5377 yeast is a ROS-resistant yeast strain. The standard yeast strain (W303) and the KNU5377 strain were exposed to 10 mM H_2O_2 for different times (0, 30, 60, 90 and 120 min). The aliquots were spotted on YPD agar plates, and were incubated at 30°C in order to determine their survival rates. (**B**) Wild-type flies are equally resistant to both normal and ROS-resistant yeasts. The adult male flies were subjected to natural infection with W303 or KNU5377. (**C**) IMD/NF-κB pathway mutant flies are susceptible to KNU5377 but not to W303. The IMD/NF-κB pathway mutant flies (*Dredd*^{B118}, *key*^{1} and *Relish*^{E20}) and the Toll/NF-κB pathway mutant flies (*spz*^{rm7} and *Dif*^{1}) were subjected to natural infection with W303 or KNU5377. (**D**) Flies exhibiting impaired regulation of the Toll pathway showed wild-type level resistance against KNU5377. Loss-of-function flies for Toll pathway (*J4* and *Pelle-RNAi/+; Da-GAL4/+*) or gain-of-function flies for Toll pathway (*cact*^{A2}) were subjected to natural infection with W303 or KNU5377. The *Pelle-RNAi/+; Da-GAL4/+* flies used in this study showed severely reduced level of infection-induced *Drosomycin* gene expression following systemic infection (data not shown). The flies exhibiting impaired potential for both Toll and IMD pathways (*Dredd; Pelle-RNAi/+; Da-GAL4/+*) were also used in this experiment. These flies showed similar immune susceptibility to that of flies carrying IMD pathway mutation alone. In all cases, survival in three or more independent cohorts of about 25 flies each was monitored over time. Results are expressed as the means ± s.d. ($P < 0.05$).

we also checked the KNU5377-induced mortality using the flies carrying Dif and Dorsal double mutation (*J4*), mutant flies exhibiting constitutive activation of Dif and Dorsal (*cact*^{A2}) and the knockdown flies for Toll pathway generated by introducing *Pelle-RNAi* using ubiquitously expressing *Daughterless* (*Da*)-GAL4 driver (*Pelle-RNAi/+; Da-GAL4/+*). In all cases, the impaired regulation of the Toll pathway (either gain-of-function or loss-of-function) showed wild-type

resistance (Figure 1D). Furthermore, the flies exhibiting reduced potential for both Dif/Dorsal-mediated Toll and Relish-mediated IMD pathway (*Dredd*^{B118}*; Pelle-RNAi/+; Da-GAL4/+*) showed similar immune susceptibility to that of flies carrying IMD pathway mutation alone (*Dredd*^{B118}, *key*^{1} or *Relish*^{E20}) (Figure 1C and D). This result is consistent with that NF-κB activity in the epithelia is controlled primarily via the IMD/NF-κB pathway but not via the Toll/NF-κB pathway (Ferrandon *et al*, 1998; Tzou *et al*, 2000; Onfelt Tingvall *et al*, 2001; Ha *et al*, 2005b). To exclude possible crosstalk between NF-κB activation and ROS production in the gut, we tested whether the ROS production or ROS-generating Duox enzyme expression is affected in the gain-of-function or loss-of-function mutant flies of NF-κB pathways. The result showed that infection-induced ROS production and Duox induction were not significantly affected in any of the tested NF-κB pathway mutant flies (Supplementary Figure 1). Conversely, Duox-RNAi flies exhibiting reduced infection-induced ROS production showed normal NF-κB target gene activation (Supplementary Figure 2). These results strongly suggest that NF-κB-dependent immunity and ROS-dependent immunity function independently as two separate defense systems but they play complementary roles in gut immunity. Furthermore, our results demonstrate that IMD/NF-κB pathway is essential for host protection against gut infection with ROS-resistant microbes, but not with normal ROS-sensitive microbes.

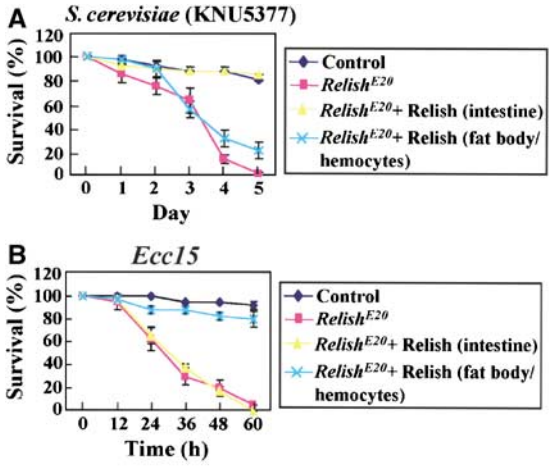

Figure 2 The susceptibility of *Relish*^{E20} flies to natural KNU5377 infection can be ameliorated via the re-introduction of *Relish* in the intestine but not in the fat body. For the rescue experiment, the *Relish*^{E20} flies were crossed with flies carrying *UAS-Relish*. The *cad-GAL4* and *c564-GAL4* drivers were used for intestine-specific and fat body/hemocyte-specific *Relish* expression, respectively. The genotypes of the flies used in this study were as follows: control (*cad-GAL4/+*); *Relish*^{E20} (*cad-GAL4/+; Relish*^{E20}); *Relish*^{E20} + Relish (intestine) (*cad-GAL4/UAS-Relish; Relish*^{E20}); *Relish*^{E20} + Relish (fat body/hemocytes) (*c564-GAL4/UAS-Relish; Relish*^{E20}). Natural gut infection (**A**) and septic infection (**B**) were performed with KNU5377 and *Erwinia carotovora carotovora 15* (Ecc15), respectively. In all cases, survival in three or more independent cohorts of about 25 flies each was monitored over time. Results are expressed as the means ± s.d. ($P<0.05$).

The gut IMD/NF-κB pathway, but not the systemic IMD/NF-κB pathway, is required for host protection from the gut infection with ROS-resistant microbes

In order to corroborate that the observed increase in KNU5377-induced mortality in the IMD/NF-κB pathway mutant flies was due to a lack of intestinal NF-κB pathway potential, we examined the effect of tissue-specific re-establishment of *Relish* expression on the survival of *Relish*^{E20} using two different tissue-specific GAL4 drivers. We used the *caudal (cad)-GAL4* driver for the re-introduction of *Relish* expression in the intestine because cad expression is effectively restricted to the posterior midgut and proventriculus (Mlodzik and Gehring, 1987). Cad is also expressed in the salivary glands and ejaculatory duct (Ryu *et al*, 2004), but not in the fat body as demonstrated by green fluorescence protein (GFP) expression pattern in *cad-GAL4/UAS-EGFP* flies (data not shown). To introduce *Relish* expression in the fat body/hemocytes (the main immune tissue of systemic immunity), we used the *c564-GAL4* driver. The *c564-GAL4* strain did not express GAL4 in the intestine as determined by GFP expression patterns in the flies carrying *c564-GAL4/UAS-EGFP* (data not shown). Importantly, the re-introduction of *Relish* expression primarily in the intestines of *Relish*^{E20} mutant flies (flies carrying *UAS-Relish/cad-GAL4; Relish*^{E20}), but not in the fat body/hemocytes of *Relish*^{E20} mutant flies (flies carrying *UAS-Relish/c564-GAL4; Relish*^{E20}), resulted in a dramatic upswing in the survival rates after the ingestion of ROS-resistant KNU5377 strain (Figure 2A). In a control experiment, the re-introduction of *Relish* in the *Relish*^{E20} by *c564-GAL4*, but not by *cad-GAL4*, efficiently protected host in the case of systemic infections (Figure 2B). This result showed that the IMD/NF-κB pathway is required in a tissue-specific manner depending on the route of infection and that the survival of the flies during KNU5377 invasion is dependent specifically on the intestinal IMD/NF-κB pathway.

ROS-removing activity can act as a virulence factor to the host lacking IMD/NF-κB pathway potential

The fact that KNU5377 is not a modified food-type yeast but instead an environmental isolate resistant to various stresses raises doubts as to whether the pathogenicity of this microbe is solely or mainly attributable to its ROS resistance. To further confirm that microbe's capacity for ROS resistance such as ROS-removing activity can be a major virulence factor to the host lacking NF-κB pathway potential, we engineered normal bacteria to overexpress a single ROS-removing enzyme, which would confer a higher potential pathogenicity due to increased ROS resistance. We used *Salmonella enterica* serotype *Typhimurium* (SL1344) and SL1344 overexpressing antioxidant *KatN* gene (SL1344-KatN) for natural gut infection. The *KatN* gene is one of the candidate genes responsible for *Salmonella* virulence, encoding a non-haem catalase responsible for ROS resistance (Robbe-Saule *et al*, 2001). To test whether *KatN* is involved in the removal of host's intestinal ROS, we measured the *in vivo* intestinal ROS level following SL1344-KatN infection. The result showed that infection-induced intestinal ROS level was significantly lower following SL1344-KatN infection, compared to that following SL1344 infection (Figure 3A). This result clearly showed that the bacterial virulent genes such as antioxidant enzyme *KatN* can efficiently antagonize the microbicidal ROS at the organism level. When we fed NF-κB pathway mutant flies on either the SL1344 strain or the SL1344-KatN, we observed high mortality levels in the flies

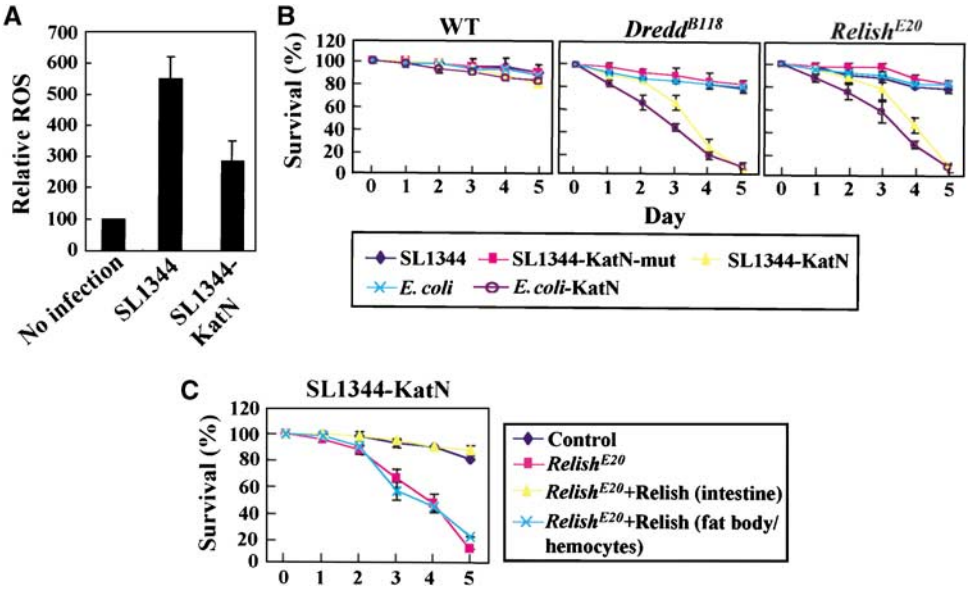

Figure 3 ROS-removing activity can act as a virulence factor to the host lacking IMD/NF-κB pathway potential. (**A**) KatN-overexpressing *Salmonella* can significantly decrease infection-induced host's ROS level. The total *in vivo* intestinal ROS levels were quantified (Ha *et al*, 2005a) with flies both before and after natural infection with control *Salmonella* (SL1344) or KatN-overexpressing *Salmonella* (SL1344-KatN). The ROS level in the uninfected control intestine was taken arbitrarily to be 100, and the results are presented as relative levels. Results are expressed as the mean and the standard deviations of three different experiments. (**B**) IMD/NF-κB pathway mutant flies are susceptible to bacteria overexpressing the *Salmonella* KatN catalase. Natural infection was performed with *Salmonella enterica* serotype *Typhimurium* (SL1344), SL1344 overexpressing *Salmonella* catalase, KatN (SL1344-KatN), SL1344 overexpressing mutant form of KatN (SL1344-KatN-mut), *E. coli* DH5α strain (*E. coli*) and DH5α strain overexpressing KatN (*E. coli*-KatN). (**C**) The susceptibility of *Relish*^{E20} flies to natural SL1344-KatN infection can be greatly ameliorated via the re-introduction of *Relish* in the intestine. The genotypes of the flies used in this study are described in Figure 2. In all cases, survival in three or more independent cohorts of about 25 flies each was monitored over time. Results are expressed as the means ± s.d. ($P<0.05$).

fed on SL1344-KatN strain (Figure 3B). No significant mortality was observed in the NF-κB pathway mutant flies fed on either the SL1344 strain or the SL1344 overexpressing mutant form of KatN (SL1344-KatN-mut) (Figure 3B). We also observed that overexpression of *KatN* gene is sufficient to render non-pathogenic *Escherichia coli* strain (DH5α) highly virulent to NF-κB pathway mutant flies (Figure 3B). Furthermore, the remarkable levels of *Salmonella* KatN-induced mortality seen in the *Relish*^{E20} flies were completely abolished as a result of the re-introduction of the *Relish* gene expression in the intestine (Figure 3C). Taken together, these results demonstrate that intestinal NF-κB-dependent innate immunity plays an essential role in protecting the host against attacks by pathogens resistant to the ROS-dependent innate immunity.

Gut AMP is required for host protection against gut infection by ROS-resistant microbes

We next investigated the molecular mechanism by which the intestinal IMD/NF-κB pathway protects the host from ROS-resistant pathogens. In *Drosophila* epithelia, the IMD/NF-κB pathway is believed to be essential for the full expression of immune effector genes, including AMPs (Ferrandon *et al*, 1998; Tzou *et al*, 2000; Onfelt Tingvall *et al*, 2001). In the case of systemic infections, the importance of AMP has been supported by the observation that constitutive expression of a single AMP can restore resistance to systemic infection to the wild-type level in Toll and IMD pathway mutants (Tzou *et al*, 2002). Although the epithelial AMPs are believed to constitute an important host defense system that inhibits the onset of local microbial proliferation in *Drosophila* (Brey *et al*, 1993; Ferrandon *et al*, 1998; Tzou *et al*, 2000; Onfelt Tingvall *et al*, 2001; Ryu *et al*, 2004), the exact *in vivo* role of epithelial AMPs has not yet been demonstrated at an organism level owing to the lack of suitable experimental models. We questioned if the high level of pathogen-induced mortality in the IMD/NF-κB pathway mutant flies was due to the absence of NF-κB-dependent local AMP expression, which would ostensibly result in microbial over-proliferation and host death in the end. We attempted to ameliorate the survival rates of *Dredd*^{B118} flies by inducing the tissue-specific expression of the AMP *Cecropin* (*Cec*) *A1* gene in the intestine. The *Drosophila Cec* gene was selected for this experiment because Cec exhibits broad microbicidal activity against both bacteria and yeast (Gazit *et al*, 1994; Ekengren and Hultmark, 1999) and because the *Cec* gene is also rapidly induced in the intestine as the result of natural gut infection with yeasts via the IMD/NF-κB pathway (Figure 4A). Our *in vitro* antimicrobial activity assay revealed that both KNU5377 and W303 strains were equally susceptible to low concentrations of synthetic Cec A1 (Figure 4B) although the KNU5377 strain exhibited a much higher resistance to ROS than the W303 strain (Figure 1A). These results demonstrate that KNU5377 has different *in vitro* sensitivities to two distinct immune effectors, ROS and AMP. Consistently, both SL1344 and ROS-resistant SL1344-KatN strains were also equally susceptible to synthetic Cec A1 (Figure 4C). Our *in vivo* rescue experiment revealed that intestine-specific Cec expression in the *Dredd*^{B118} flies (*Dredd*^{B118}*;UAS-Cec/cad-GAL4*) was sufficient to confer protection against natural KNU5377 or SL1344-KatN infection in the host lacking a functional IMD/NF-κB pathway (Figure 4D and E).

Gut IMD/AMP system is required for the efficient clearance of ROS-resistant microbes in the intestine

In order to further verify that the natural infection-induced mortality of *Dredd*^{B118} flies was due to uncontrolled microbial proliferation in the absence of AMPs and that the host protection seen in *Dredd*^{B118}*;UAS-Cec/cad-GAL4* flies was due to Cec-mediated antimicrobial activity, we attempted to assess the persistence of the ROS-resistant microbes in the intestines of the control, *Dredd*^{B118} and *Dredd*^{B118}*; UAS-Cec/cad-GAL4* flies. First, it was shown that the KNU5377 counts in the intestines of *Dredd*^{B118} flies were ~100 times higher than those measured in the control flies (Figure 5A and Supplementary Figure 3). Next, the levels of KNU5377 found in the intestines of *Dredd*^{B118} flies were reduced to control levels via the introduction of intestinal *Cec* expression into the *Dredd*^{B118} flies (Figure 5A and Supplementary Figure 3). The results are consistent with that the marked KNU5377 proliferation was due to the absence of AMPs in the intestines of the *Dredd*^{B118} flies. In a separate experiment, we used the GFP-tagged *E. coli* DH5α (*E. coli*-GFP) or *E. coli*-GFP overexpressing KatN (*E. coli*-KatN-GFP), which allowed us to follow in real time *in vivo* microbial persistence in the intestines of the hosts. In the control flies, we observed no significant microbial persistence following the ingestion of either *E. coli*-GFP or *E. coli*-KatN-GFP (Figure 5B). However, in the NF-κB pathway mutant flies, a marked difference in microbial persistence was observed between *E. coli*-GFP and *E. coli*-KatN-GFP. In the case of *E. coli*-GFP ingestion, we observed no significant persistence of the bacteria in the intestines of the *Dredd*^{B118} flies as seen with the wild-type flies (Figure 5B). This result is consistent with the data in Figure 3B indicating that no significant *E. coli*-induced mortality was detected in either the control flies or the NF-κB pathway mutant flies. In contrast, in the case of *E. coli*-KatN-GFP ingestion, marked microbial persistence was detected in the intestines of the *Dredd*^{B118} flies (Figure 5B). Importantly, such high level of *E. coli*-KatN persistence could be completely removed by intestine-specific expression of Cec (*Dredd*^{B118}*; UAS-Cec/cad-GAL4*) (Figure 5B). Taken together, these results demonstrate that activation of the intestinal NF-κB pathway and subsequent local AMP expression play a critical role in host survival by limiting the proliferation of virulent pathogenic strains that are able to circumvent the host's ROS-dependent innate immune system.

Introduction of the ROS-resistant bacteria in the gut induces severe damage to intestinal epithelial cells lacking NF-κB/AMP pathway

To determine the direct cause of death in NF-κB-mutant flies exposed to ROS-resistant microbes, we performed histological examinations of the gut tissue following natural infection. No significant gut pathology was observed in the control flies following the ingestion of either *E. coli* or *E. coli*-KatN (Figure 6A and B). In the NF-κB mutant *Relish*^{E20} flies however, a dramatic difference in gut morphology was observed between *E. coli* and *E. coli*-KatN. Ingestion of *E. coli*-KatN, but not

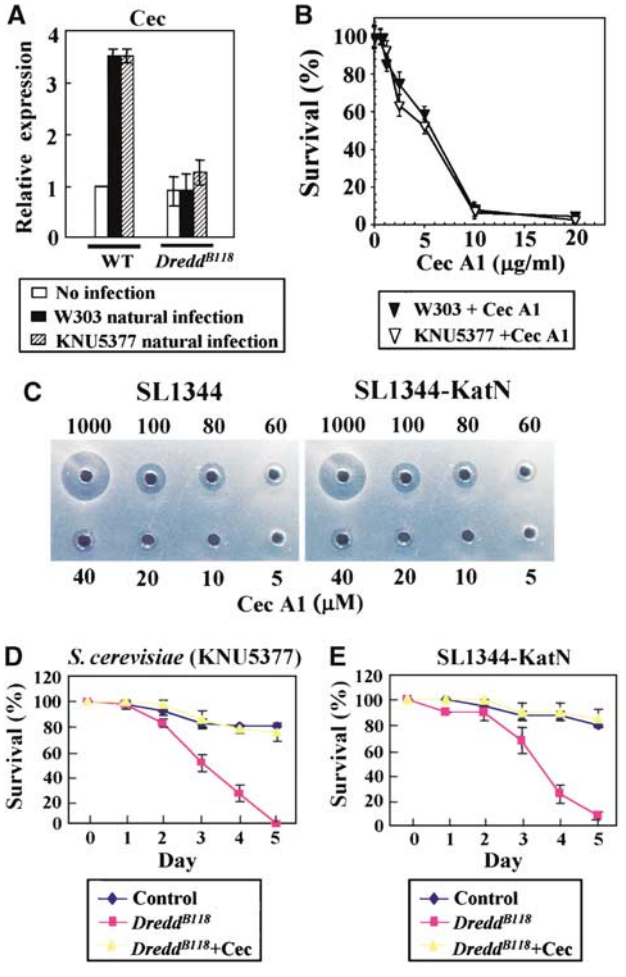

Figure 4 Intestinal AMP expression is indispensable for host protection from attack of ROS-resistant pathogens. (**A**) Natural yeast infection induces Cecropin (*Cec*) expression in the midgut via IMD/NF-κB pathway. Wild-type flies (WT) or IMD/NF-κB pathway mutant flies (*Dredd*^{B118}) were subjected to natural infection (0 and 12 h) with either the W303 or KNU5377 strains. Quantitative real-time PCR analysis of *Cec* gene transcription was performed using dissected midguts. *Cec* expression in the tissues of uninfected WT flies was taken arbitrarily as 1, and the results are shown as relative expressions. Results are expressed as the means ± s.d. ($P<0.05$) of three different experiments. (**B**) Both KNU5377 and W303 strains are found to be equally susceptible to low concentrations of synthetic Cec A1 peptide. Yeast cells were incubated with serially diluted Cec A1 peptide at 28°C for 18 h. Antifungal activity was performed as described in Materials and methods. Results are expressed as the means ± s.d. ($P<0.05$) of three different experiments. (**C**) Both SL1344 and SL1344-KatN strains are equally susceptible to synthetic Cec A1. To measure the antibacterial activity, inhibition zone assay were performed with serially diluted Cec A1, as described in Materials and methods. (**D**) The susceptibility of the *Dredd*^{B118} flies to natural KNU5377 infection can be dramatically ameliorated by the ectopic expression of Cec A1 in the intestine. For the rescue experiment, *Dredd*^{B118} flies were crossed with flies carrying *UAS-Cec A1*. The *cad-GAL4* driver was used for intestine-specific Cec expression. The genotypes of the flies used in this study were as follows: control (*cad-GAL4/+*); *Dredd*^{B118} (*Dredd*^{B118}*; cad-GAL4/+*); *Dredd*^{B118} + Cec (*Dredd*^{B118}*; cad-GAL4/UAS-Cec*). Natural infection was performed with KNU5377. Survival in three or more independent cohorts of about 25 flies each was monitored over time. Results are expressed as the means ± s.d. ($P<0.05$). (**E**) The susceptibility of IMD/NF-κB pathway mutant flies to ROS-resistant *Salmonella* infection can be dramatically ameliorated by ectopic *Cec A1* expression in the intestine. The genotypes of the flies used in this study are shown in panel (D). Natural infection was performed with SL1344-KatN. Survival in three or more independent cohorts of about 25 flies each was monitored over time. Results are expressed as the means ± s.d. ($P<0.05$).

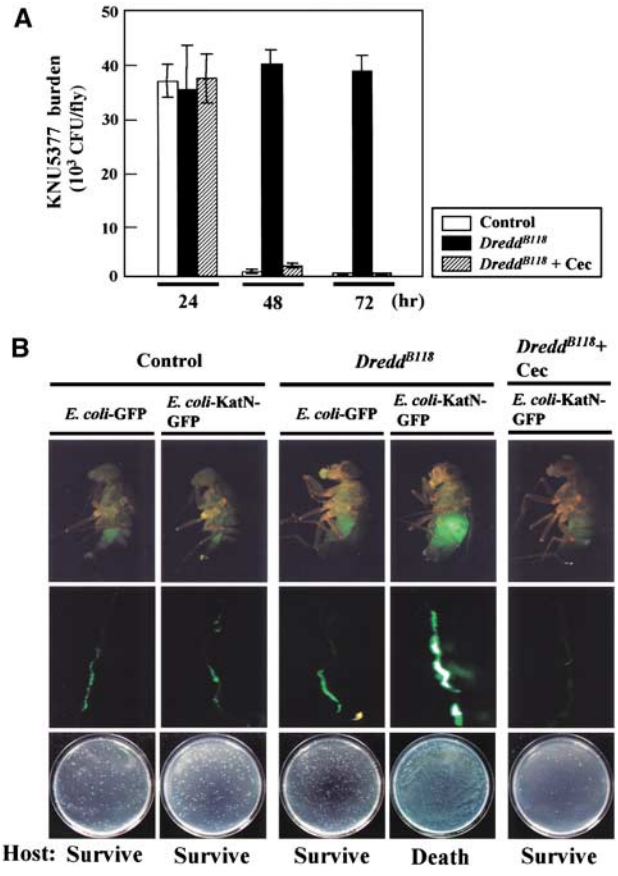

Figure 5 Intestinal NF-κB-dependent AMP expression is required for the inhibition of the proliferation of ROS-resistant virulent pathogens. (**A**) KNU5377 persists in the guts of the *Dredd*^{B118} flies, but not in the guts of either the wild-type or *Dredd*^{B118} flies expressing intestinal Cec A1. In order to construct a rescue line, *Dredd*^{B118} flies were crossed with flies carrying *UAS-Cec A1*. The genotypes of the flies used in this study are listed in Figure 4D. Natural infection was performed with G418-resistant KNU5377. KNU5377 persistence was measured by plating appropriate dilutions of homogenates of five surface-sterilized intestines, which had been collected at different times after infection. Microbes were grown on YPD plates containing G418 (200 μg/ml). The number of CFUs per adult intestine obtained at each time point after infection represents the means ± s.d. ($P<0.05$) of three different experiments. (**B**) The high persistence of ROS-resistant bacteria can be controlled by NF-κB/AMP pathway in the intestine of live *Drosophila*. *E. coli* overexpressing KatN, but not *E. coli* that does overexpress KatN, persists in the guts of *Dredd*^{B118} flies. GFP-tagged *E. coli* DH5α strain (*E. coli*-GFP) and *E. coli*-GFP overexpressing KatN (*E. coli*-KatN-GFP) were used for the *in vivo* real-time analysis of bacterial persistence. The genotypes of the flies used in this study are shown in Figure 4D. Representative images of naturally infected flies (upper panels), dissected intestines (middle panels) and representative plates of *E. coli* recovered from the intestines (lower panels), at 72 h after infection, are shown.

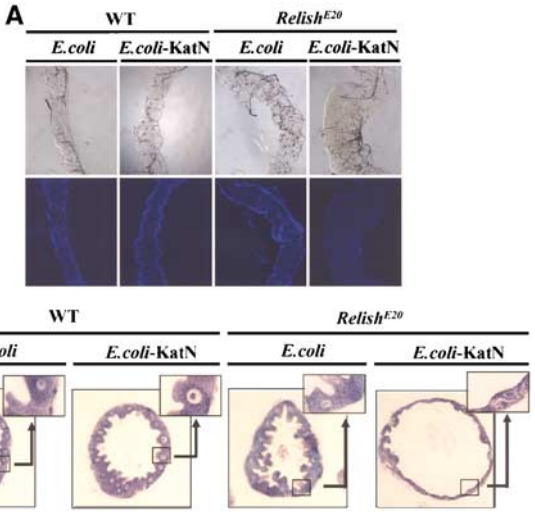

Figure 6 The high persistence of ROS-resistant bacteria causes a severe abnormality in the intestine of NF-κB mutant *Drosophila*. (**A, B**) Wild-type and *Relish* mutant flies were naturally infected with *E. coli* or *E. coli*-KatN. Midguts were dissected out at 72 h post-infection and examined under light and fluorescence microscopy (A). Nuclear staining was performed with DAPI. Transverse sections of the midgut were stained with toluidine blue and analyzed under light microscopy (B). Magnification is × 400 in each panel. Insets show high magnification (× 1000) of the boxed area.

normal *E. coli*, induced severe morphological abnormalities. Specifically, the midgut of the *Relish*^{E20} flies became visibly swollen, suggesting a significant damage to the gut at the tissue or cell level (Figure 6A). Nuclear staining of the midgut epithelial cells showed that the epithelial cells of the *Relish*^{E20} disappeared or were severely damaged compared to the controls (Figure 6A). Consistently, cross-sections of the midgut revealed a morphological alteration of the columnar structure and degeneration of epithelial cells (Figure 6B). Interestingly, although the visceral musculature appeared to be intact, actin staining showed that *E. coli*-KatN ingestion induced a loss of typical intestinal cell shape as the cells adopted a flat morphology in the case of the *Relish*^{E20} (Figure 7A). Consistent with the *in vivo* persistence experiment (Figure 5B), 4′,6′-diamidino-2-phenylindole (DAPI) staining showed that high numbers of *E. coli*-KatN were observed only in the intestinal lumen of *Relish*^{E20} (Figure 7A). As no bacteria were detected in the *Relish*^{E20} hemolymph after ingestion with *E. coli-KatN* (data not shown), the systemic infection from the microbial invasion into the hemocoel by crossing gut epithelia does not seem to be the cause of host mortality. Ingestion of *E. coli*-KatN induced a statistically significant change in apoptosis of *Relish*^{E20} intestinal cells, as judged by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay (Figure 7B). Interestingly, severe gut abnormalities of *Relish*^{E20} flies were only observed following ingestion of *E. coli*-KatN, but not following a systemic infection of the same bacteria (Supplementary Figure 4). Taken together, these results indicate that marked persistence of the ROS-resistant bacteria in the gut due to the absence of NF-κB/AMP pathway causes severe damage to gut epithelial cells and subsequently results in host death.

Discussion

We have demonstrated that the intestinal NF-κB activation and subsequent local AMP induction are key elements of gut immunity in *Drosophila*. Some earlier studies in mammals have also described the *in vivo* protective role of mammalian AMPs against certain invasive pathogenic infections occurring in the barrier epithelia including the intestine and the skin (Wilson *et al*, 1999; Nizet *et al*, 2001; Salzman *et al*, 2003). In *Drosophila* gut immunity, it has been shown that ROS-mediated antimicrobial response is essential for host

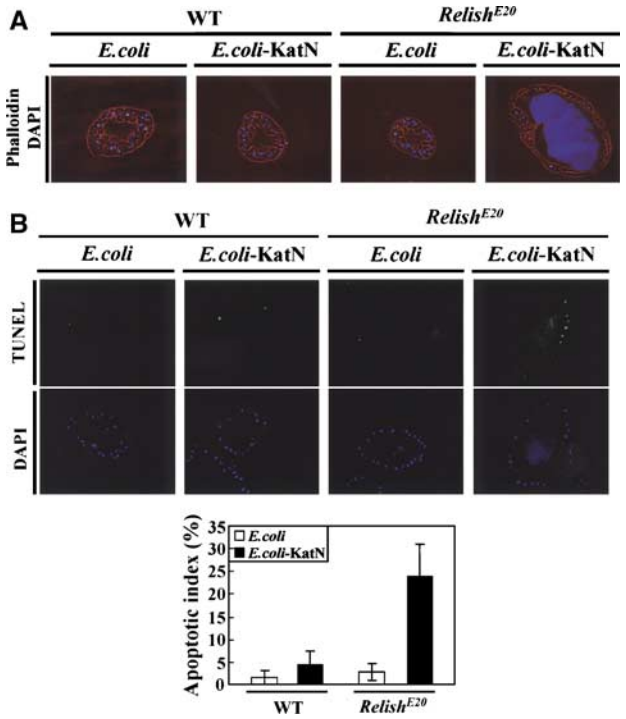

Figure 7 Ingestion of ROS-resistant bacteria induces loss of typical intestinal cell shape and the apoptosis of $Relish^{E20}$ intestinal cells. Wild-type and *Relish* mutant flies were naturally infected with *E. coli* or *E. coli*-KatN. Midguts were dissected out at 72 h post-infection. Transverse sections of the midgut were used in this experiment. (**A**) Actin staining of the midgut cells. The F-actin was stained with Alexa 568 phalloidin (red) and nuclear staining was performed with DAPI (blue). Tissues were examined under fluorescence microscopy and merged images were presented. (**B**) Apoptosis of the midgut cells. DNA strand breaks of the apoptotic cells were detected by incorporation of the fluorescein-labeled nucleotide. Nuclear staining was performed with DAPI (blue). The samples were examined under fluorescence microscopy. Intestinal cells were randomly selected for apoptosis index analysis and the number of apoptotic cells was calculated. Apoptosis index was determined by dividing the number of cells by total number of cells and multiplying by 100. Values represent the means ± s.d. ($P<0.05$) of three independent experiments.

survival during gut infection (Ha *et al*, 2005a, b). In addition to oxidant-dependent immunity, phagocytosis by macrophages also plays an important role in a gut infection model (Kocks *et al*, 2005). Our present study revealed that in the *Drosophila* gastrointestinal tract, NF-κB/AMP-dependent innate immunity is normally dispensable but provisionally crucial in case the host encounters ROS-resistant microbes. Although the precise mechanism by which ROS-resistant microbes induce epithelial cell damages remains to be investigated, we can speculate that high numbers of local microbes may produce metabolites toxic to the gut epithelia. Alternatively, it is also possible that excess chronic inflammation due to persistent microbes may cause host gut pathology similar to host immune effector-induced metabolic collapse observed in a *Salmonella*-infected *Drosophila* model (Brandt *et al*, 2004).

It should be noted that yeast and *E. coli* are not pathogens for the fly in normal situations and that manipulations to render these microbes ROS resistant may not directly reflect natural infection pathways in the animal. However, as ROS are known to be involved in many of the complex interactions between the invading microorganisms and the host (Miller and Britigan, 1997), our approach will likely be a relevant method in understanding the integrative relationship between gut immunity and microbial pathogenesis. Arthropod gut immunity during host–pathogen interactions is particularly interesting because the majority of deadly arthropod-transmitted pathogens/parasites causing illnesses such as malaria, plague, typhus and lyme disease have evolved to use the host's gut as a route of transmission (Schneider, 2000). Within the context of pathogen survival strategies, microbial pathogens must evade or counteract innate immune effectors such as ROS and AMPs in order to disseminate and cause diseases (Islam *et al*, 2001; Fang, 2004; Sansonetti, 2004; Bader *et al*, 2005). In a constant competition for survival, the pathogen and the host have developed strategies to overcome the other. Along with the highly efficient microbicidal ROS, the *Drosophila* gastrointestinal tract has been shown to express at least seven different IMD/NF-κB-dependent AMPs (Tzou *et al*, 2000), each exhibiting a distinct spectrum of *in vitro* antimicrobial activity (Hertu *et al*, 1998). In this context, we propose that the different spectra of microbicidal activity encompassed by ROS and AMPs may provide the necessary versatility to the *Drosophila* gastrointestinal innate immune system to ward off microbial infections. Furthermore, our findings suggest that the diversification of intestinal innate immune effectors into ROS and AMP systems might have been driven by selective pressures exerted on the *Drosophila* gastrointestinal tract by its constant interactions with a series of different microbial species that employ different immune-evasion strategies.

Materials and methods

Natural gut infection

Natural gut infection experiments with various microbes were performed as described previously (Ha *et al*, 2005a, b). Briefly, adult flies were dehydrated for 2 h without food and then transferred into a vial containing filter paper hydrated with 5% sucrose solution contaminated with concentrated microbe solution ($\sim 10^{10}$ colony forming units (CFU)/ml). Microbial culture during log growth phase ($OD_{600}=1$) was used for all experiments. Filter papers were changed everyday. The flies that fed on sucrose only were used as a control. All animals were incubated at 25°C. Survival rates were expressed as means and standard deviations from at least three independent experiments. In the case of *Saccharomyces cerevisiae*, standard W303 strain and ROS-resistant KNU5377 strain were used (Kwak *et al*, 2003). In the case of *Salmonella*, *Salmonella enterica* serotype *Typhimurium* (SL1344), SL1344 overexpressing *Salmonella* catalase, KatN (SL1344-KatN), and SL1344 overexpressing mutant form of KatN (SL1344-KatN-mut) were used (Robbe-Saule *et al*, 2001). The plasmid pOM1-GFP (Basset *et al*, 2003) was used to transform *E. coli* DH5α strain to obtain *E. coli*-GFP. The plasmid pQE60-G57 (expressing a C-terminal 6xHis fusion to the KatN gene under control of isopropylthio-β-D-galactoside (IPTG)-inducible promoter) (Robbe-Saule *et al*, 2001) was used to transform *E. coli*-GFP bacteria to obtain *E. coli*-KatN-GFP. In experiments using *E. coli*-KatN-GFP strain, KatN protein was induced in the presence of 1 mM IPTG for 3 h. Systemic septic infection was performed as described previously (Ryu *et al*, 2004).

Constructs and fly strains

The entire open reading frame of the Cec A1 was subcloned into the pUAST vector (Brand and Perrimon, 1993) to obtain the *UAS-Cec* construct. This construct was then used to generate transgenic flies by P element-mediated transformation (Rubin and Spradling, 1982). The fly stocks used in this study have been described previously: spz^{rm7} (Lemaitre *et al*, 1996); $Relish^{E20}$ (Hedengren *et al*, 1999); key^{1} (Rutschmann *et al*, 2000); *J4* (Meng *et al*, 1999); $cact^{A2}$ (Lemaitre

et al, 1996); *UAS-Relish* (Hedengren *et al*, 1999); *UAS-Duox-RNAi* (Ha *et al*, 2005a); *Da-GAL4* (Giebel *et al*, 1997); *c564-GAL4* (Harrison *et al*, 1995; Takehana *et al*, 2004) and *cad-GAL4* (Moreno and Morata, 1999).

In vitro antifungal assay

The yeast (W303 or KNU5377), grown at 28°C in YPD (dextrose 2%, peptone 1%, yeast extract 0.5%) medium, were seeded on 96-well microtiter plates at a density of 2×10^3 cells per well in 100 μl of YPD medium. Cells were mixed with 10 μl of the serially diluted synthetic Cec A1 peptide solutions. The peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry (Lee *et al*, 2003). The cell suspension was incubated at 28°C for 18 h. After incubation of cell suspension, 5 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (5 mg/ml MTT in phosphate-buffered saline (PBS), pH 7.4) was added to each well and the plates were incubated at 37°C for 4 h. A 30 μl portion of 20% (w/v) SDS solution containing 0.02 M HCl was added, and then the plates were incubated at 37°C for 16 h to dissolve the formazan crystals that had formed. The turbidity of each well was measured at 580 nm by a microtiter ELISA reader (Molecular Devices Emax, California, USA) (Jahn *et al*, 1995).

In vitro antibacterial assay

The antibacterial activity was assessed by an inhibition zone assay using thin agarose plates seeded with test bacteria (Brey *et al*, 1993). Each well of plates received 10 μl of the serially diluted synthetic Cec A1 peptide solutions. All detection plates were placed at 4°C for 30 min and then incubated at 30°C for 24 h, at which time inhibition zones were scored. A zone of inhibition (>1 mm from the edge of the well) of bacterial growth, in which the agarose appeared clear, was considered indicative of antibacterial activity.

Measurement of colony-forming units

For the comparison of microbial burden in the intestine, adult male flies (control flies, $Dredd^{B118}$ flies and $Dredd^{B118}$ flies overexpressing Cec in the intestine) were naturally infected with various microbes (*E. coli*-GFP, *E. coli*-KatN-GFP and KNU5377 yeast). At the time point after infection (24, 48 and 72 h), flies were collected and the intestines were dissected. These intestines were first rinsed in water, dipped in 70% (vol/vol) ethanol for sterilization and then diluted in sterilized PBS (pH 7.4) solution. In the case of KNU5377 detection, surface-sterilized intestines were homogenized and spread onto YPD plate containing G418 (200 μg/ml) by appropriate dilutions (1/4 and 1/40). *E. coli*-GFP and *E. coli*-KatN-GFP were detected in LB-spectinomycin (100 μg/ml) plates and LB-spectinomycin (100 μg/ml)/ampicillin (100 μg/ml) plates, respectively. The number of CFUs per adult intestine obtained at each time point represents the means of at least three independent measurements.

In vitro ROS resistance assay

The W303 and KNU5377 yeast strain were grown in YPD medium to an OD_{600} of 1.0, and hydrogen peroxide (H_2O_2) was added to a final concentration of 10 mM. To check their survivals, aliquots of yeast were removed at timed intervals (0, 30, 60, 90 and 120 min), spotted on YPD agar plate and incubated at 30°C for 36 h.

Real-time PCR analysis

To quantify the amount of gene expression, fluorescence real-time PCR was performed with the double-stranded DNA dye, SYBR Green (Perkin Elmer, Boston, MA). Primer pairs for Cec (sense, 5′-ATG AAC TTC TAC AAC ATC TTC G-3′; antisense, 5′-GGC AGT TGC GGC GAC ATT GGC G-3′), Dipt (sense, 5′-GGC TTA TCC GAT GCC CGA CG-3′; antisense, 5′-TCT GTA GGT GTA GGT GCT TCC-3′), dDuox (sense, 5′-TAG CAA GCC GGT GTC GCA ATC AAT-3′; antisense, 5′-ACG GCC AGA GCA CTT GCA CAT AG-3′) and control *Rp49* (sense, 5′-AGA TCG TGA AGA AGC GCA CCA AG-3′; antisense, 5′-CAC CAG GAA CTT CTT GAA TCC GG-3′) were used to detect target gene transcripts. SYBR Green analysis was performed on an ABI PRISM 7700 system (PE Applied Biosystems) according to the manufacturer's instructions. All samples were analyzed in triplicate, and the levels of detected mRNA were normalized to control *Rp49* mRNA values. The normalized data were used to quantify the relative levels of a given mRNA according to cycling threshold (ΔC_t) analysis (Leulier *et al*, 2003). The *Cec* expression in the intestine of uninfected wild-type flies was taken arbitrarily as 1, and the results are presented as relative expression levels.

Histological and immunohistochemical analyses

The midguts were dissected out at 72 h after natural bacterial infection and fixed in 4% paraformaldehyde. After staining with DAPI, the midgets were examined with an epiflourescence microscope. Alternatively, dissected midguts were fixed in 10% neutral buffered formalin, dehydrated in an ascending series of ethanol concentrations and then embedded with paraffin. Paraffin sections (3 or 5 μm) were stained with toluidine blue and examined by standard light microscopy.

Actin was visualized with Alexa 568 phalloidin (Molecular Probes). Fragmented DNA was stained using the terminal deoxynucleotidyltransferase (TdT)-mediated dUDP nick end labeling method using the *In Situ* Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's instructions.

Measurement of in vivo ROS

The level of total *in vivo* gut ROS was quantified as described previously (Ha *et al*, 2005a).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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