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Regulation of DUOX by the Gαq-Phospholipase Cβ-Ca^{2+} Pathway in *Drosophila* Gut Immunity

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SUMMARY

All metazoan guts are in constant contact with diverse food-borne microorganisms. The signaling mechanisms by which the host regulates gut-microbe interactions, however, are not yet clear. Here, we show that phospholipase C-β (PLCβ) signaling modulates dual oxidase (DUOX) activity to produce microbicidal reactive oxygen species (ROS) essential for normal host survival. Gut-microbe contact rapidly activates PLCβ through Gαq, which in turn mobilizes intracellular Ca^{2+} through inositol 1,4,5-trisphosphate generation for DUOX-dependent ROS production. *PLCβ* mutant flies had a short life span due to the uncontrolled propagation of an essential nutritional microbe, *Saccharomyces cerevisiae*, in the gut. Gut-specific reintroduction of the *PLCβ* restored efficient DUOX-dependent microbe-eliminating capacity and normal host survival. These results demonstrate that the Gαq-PLCβ-Ca^{2+}-DUOX-ROS signaling pathway acts as a bona fide first line of defense that enables gut epithelia to dynamically control yeast during the *Drosophila* life cycle.

INTRODUCTION

All organisms are in constant contact with a large number of different types of microbes. This is especially true in the case of the gut epithelia, which control life-threatening pathogens as well as food-borne microbes. In addition to this microbe-eliminating capacity, gut epithelia also need to protect normal commensal microbes which are in a mutually beneficial relationship. Therefore, gut epithelia must be equipped to differentially operate innate immunity in order to efficiently eliminate life-threatening microbes while protecting beneficial microbes. Studies using *Drosophila melanogaster* as a genetic model have greatly enhanced our understanding of the microbe-controlling mucosal immune strategy in gut epithelia (Bischoff et al., 2006; Ha et al., 2005a, 2005b; Nehme et al., 2007; Ryu et al., 2006, 2008; Zaidman-Remy et al., 2006). Previous studies in a gut infection model using oral ingestion of pathogens revealed that the redox system has an essential role in host survival by generating microbicidal effectors such as reactive oxygen species (ROS) (Ha et al., 2005a, 2005b). In this redox system, dual oxidase (DUOX), a member of the nicotinamide adenine dinucleotide phosphate (NADP)H oxidase family, is responsible for the production of ROS in response to gut infection (Ha et al., 2005a). Following microbe-induced ROS generation, ROS elimination is assured by immune-regulated catalase (IRC), thereby protecting the host from excessive oxidative stress (Ha et al., 2005b). In addition to the redox system, the mucosal *immune deficiency* (IMD)/NF-κB signaling pathway, which leads to the de novo synthesis of microbicidal effector molecules such as antimicrobial peptides (AMPs) (Ferrandon et al., 1998; Uvell and Engstrom, 2007), has an essential complementary role to the redox system when the host encounters ROS-resistant pathogenic microbes (Ryu et al., 2006). These findings indicate that the different spectra of microbicidal activity encompassed by ROS and AMPs may provide the versatility necessary for *Drosophila* gut immunity to control microbial infections. Furthermore, in the absence of gut infection, a selective repression of IMD/NF-κB-dependent AMPs is mediated by the homeobox gene *Caudal*, which is required for protection of the resident commensal community and host health (Ryu et al., 2008). Therefore, fine-tuning of different gut immune systems appears to be essential for both the elimination of pathogens and the preservation of commensal flora.

Most studies evaluating gut immunity have been performed in an oral infection model in which the pathogens are ingested (Bischoff et al., 2006; Ha et al., 2005a, 2005b; Nehme et al., 2007; Ryu et al., 2006, 2008; Zaidman-Remy et al., 2006). However, the gut epithelia constitute the interface between the host and the microbial environment; therefore, it is likely that animals in nature have already been subjected to continuous microbial contact, even in the absence of oral infection. Thus, it is essential to determine the mechanism by which this natural and continuous microbial interaction produces ROS at a tightly controlled, yet adequate level that allows for healthy gut-microbe interactions and gut homeostasis, because deregulated generation of ROS is believed to lead to a pathophysiologic condition in the gut epithelia. Although the DUOX system is of central importance

in gut immunity, the signaling pathway(s) by which gut epithelia regulate DUOX-dependent microbicidal ROS generation are poorly understood.

Drosophila feed on microbes, and one of their most essential microbial food sources is baker's yeast, *Saccharomyces cerevisiae*. As early as 1930, yeast was discovered to be an essential nutrient source for *Drosophila* (Alpatov, 1930) and is now used as a major ingredient in standard laboratory *Drosophila* food recipes. Further, *Drosophila-Saccharomyces* interaction occurs in wild-captured *Drosophila* (Phaff et al., 1956), which suggests that this interaction is an evolutionarily ancient natural phenomenon. Although many studies have investigated the effect of yeast on *Drosophila* metabolism and aging (Min et al., 2008; Partridge et al., 2005), very few works have been reported on the effect of yeast in terms of the host immunity. Specifically, it has previously been shown that dietary yeast contributes to the cellular immune responsiveness of *Drosophila* against a larval parasitoid, *Leptopilina boulardi* (Vass and Nappi, 1998). However, the relationship between yeast and *Drosophila* gut immunity during the normal life cycle has never been closely examined. Therefore, in this study, we used a *Drosophila*-yeast model to investigate the intracellular signaling pathway by which the host mounts mucosal antimicrobial immunity, as well as the in vivo value of this pathway in the host's natural life. Through biochemical and genetic analyses, we revealed that the Gαq-mediated phospholipase C-β (PLCβ) pathway is involved in the routine control of dietary yeast in the *Drosophila* gut. PLCβ is dynamically activated in the presence of ingested yeast and subsequently mobilizes the intracellular Ca^{2+} to produce ROS in a DUOX-dependent manner. The presence of all of these signaling components of the Gαq-PLCβ-Ca^{2+}-DUOX-ROS pathway in the gut is essential to ensure routine control of dietary yeast and host fitness, which highlights the importance of this immune signaling as a bona fide first line of defense in *Drosophila*.

RESULTS

Natural *Drosophila*-Yeast Interactions Are Controlled by DUOX-Dependent Gut Immunity

Yeast is an essential nutrient source for *Drosophila* (Alpatov, 1930). In the wild, flies feed on live yeast found in decaying and fermented fruits, and most flies caught in the wild are physically associated with various strains of yeast, although they are predominantly associated with *Saccharomyces* (Phaff et al., 1956). Cornmeal-agar media supplemented with yeast, *S. cerevisiae*, is used as a standard food recipe in most laboratory-rearing conditions. In this case, the supplemented yeast was dead due to the heat treatment during food preparation (referred to as dead yeast media in the present study). Therefore, to mimic naturally occurring interactions between *Drosophila* and live yeast in the absence of experimental infection, we used food that was supplemented with live yeast (referred to as live yeast media) instead of dead yeast. Under these conditions, we investigated whether the in vivo *Drosophila*-yeast interaction is controlled through the host immune system. We found that control flies remained healthy in the live yeast media, despite the long-lasting microbial contact (Figure 1A). Host survival rates did not significantly differ between the live yeast and the dead yeast media (Figure 1A). Only a few yeast colonies (based on the number of colony-forming units, CFUs) were detected in the dissected midgut of control flies reared in the live yeast media (Figure 1B), indicating that live yeast is unable to stably colonize the gut.

To determine if previously identified mucosal immune systems such as the NF-κB-AMP pathway or the DUOX-ROS pathway are involved in the control of nutritional microbes, we examined the survival rates of flies lacking AMP or ROS production. In this experiment, we used Toll pathway mutant flies (Dif^1), IMD pathway mutant flies (Rel^{E20}), and *DUOX-RNAi* flies. The results showed that *DUOX-RNAi* flies, but not NF-κB pathway mutant flies, were short-lived in the live yeast media (Figure 1A). This lethality, however, was significantly ameliorated in the dead yeast media in which live yeast was substituted with heat-killed yeast (Figure 1A). Similar results were obtained when germ-free *DUOX-RNAi* flies were tested in the live or dead yeast media (see Figure S1 available online), confirming that yeast, but not the commensal bacteria, is involved in the host mortality under the above experimental condition. These results indicated that DUOX, but not the Toll or IMD pathways, is required for the routine control of dietary yeast. Consistent with this result, low-yeast CFUs were observed in the gut of Rel^{E20} and Dif^1 flies, whereas high-yeast CFUs were observed in the gut of *DUOX-RNAi* flies (Figure 1B). To see whether yeast was actually growing in the *DUOX-RNAi* gut, we let flies feed on the live yeast for a defined time (1 hr) and examined CFUs in the gut. The result showed that the number of living yeast in the *DUOX-RNAi* gut was comparable to that found in the control gut immediately after the yeast feeding period, in turn indicating that the feeding rates are similar between control and *DUOX-RNAi* flies (Figure 1C). To see whether ingested yeast is proliferating in the gut of *DUOX-RNAi* flies, flies fed on live yeast were transferred to a vial containing sterile dead yeast media and time course analysis for the number of CFUs was performed. The result showed that the initial CFUs of the control gut decreased to a low level starting from 24 hr postingestion, whereas the initial CFUs of the *DUOX-RNAi* gut were maintained for 48 hr postingestion and increased steadily thereafter (Figure 1C). Such observation indicates that yeast is actually growing in the gut of *DUOX-RNAi* flies, probably due to the absence of microbicidal DUOX activity. Thus, *DUOX-RNAi* flies likely died as a result of overproliferation of yeast in the gut. Taken together, these findings indicate that the DUOX-ROS system is required for the naturally occurring *Drosophila*-yeast interaction, which may be essential for host nutrition and health.

Yeast Induces DUOX-Dependent ROS in *Drosophila* S2 Cells

The results presented above indicated that ingested nutritional microbes are efficiently controlled by the DUOX system. Therefore, we used this evolutionarily ancient natural *Drosophila*-yeast interaction to investigate the intracellular signaling pathway that leads to DUOX regulation. Because the microbicidal ROS production that occurs in response to gut infections is DUOX dependent (Ha et al., 2005a), we examined whether *Drosophila* cells produce DUOX-dependent ROS in response to yeast. *Drosophila* immunocompetent S2 cells were treated with yeast extracts, and the intracellular ROS levels were evaluated using

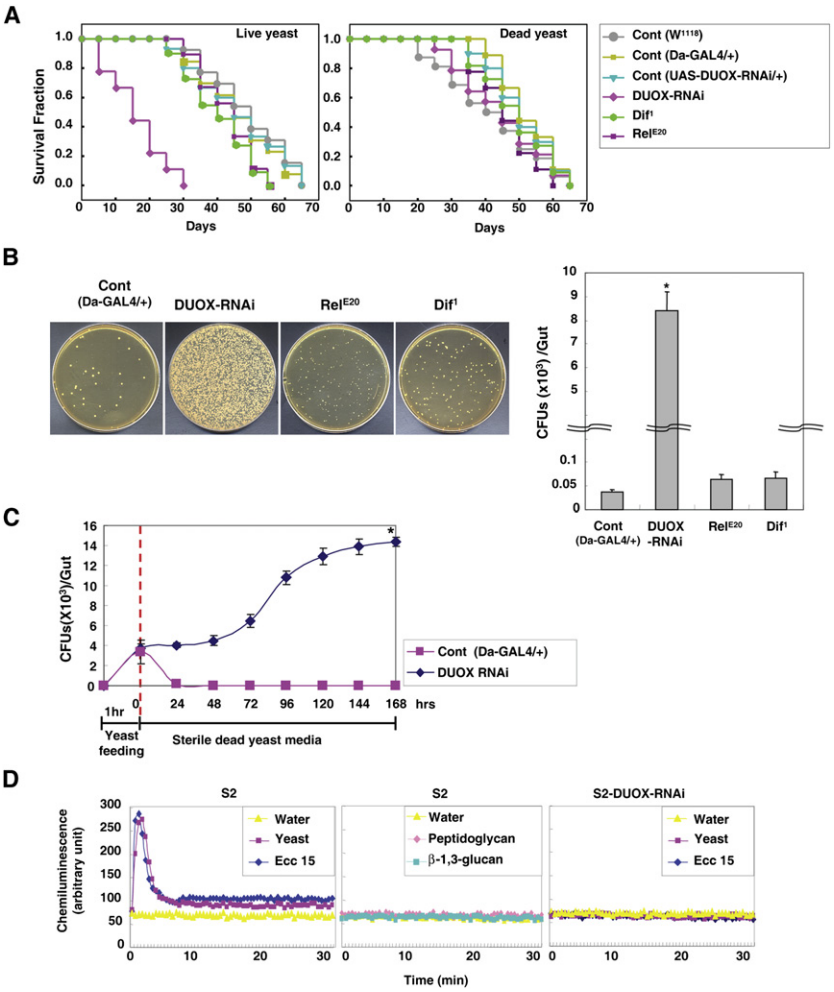

Figure 1. DUOX, but Neither Toll nor IMD Pathway, Is Required for Routine Control of Yeast and Host Survival through ROS Generation

(A) DUOX is required for normal host survival during *Drosophila*-yeast interaction. The susceptibility of flies was monitored in the absence of infection when flies were provided with food that contained either live yeast (live yeast media) or heat-killed dead yeast (dead yeast media). Survival in three or more independent cohorts comprising approximately 25 flies each was monitored over time. Log rank analysis showed a statistically significant difference in survival in live yeast media between *DUOX-RNAi* flies and w^{1118} control flies ($p < 0.0001$). The genotypes of the flies used in this study (A and B) were as follows: Cont (w^{1118}); Cont (*Da-GAL4*/+); Cont (*UAS-DUOX-RNAi*/+) Dif^{1}; Rel^{E20}; *DUOX-RNAi* (*UAS-DUOX-RNAi*/+; *Da-GAL4*/+).

(B) Yeast persists in the guts of *DUOX-RNAi* flies but not in the guts of NF-κB pathway mutant flies. Flies were maintained in live yeast media. Yeast persistence was then measured by plating appropriate dilutions of homogenates of five surface-sterilized intestines onto YPD plates. Rifampicin (100 μg/ml) was used in YPD plates because yeast was rifampicin resistant whereas commensal microbes were rifampicin sensitive. Representative plates of yeast recovered from the intestines are shown in the left panel. The number of CFUs per adult intestine (right panel) represents the means ± SD of three different experiments. Asterisk reflects value significantly different from the control at the $p < 0.001$ (ANOVA test).

(C) Yeast proliferates in the gut of *DUOX-RNAi* flies. Flies were starved for 1 hr and fed on yeast (approximately 10^8 CFUs) for 1 hr. Following yeast feeding, flies were transferred to a vial containing sterile dead yeast media and the yeast CFUs were determined at different time points. Asterisk reflects value significantly different from the control at the $p < 0.001$ (ANOVA test).

(D) Yeast induces ROS generation in a DUOX-dependent manner. S2 cells or S2 cells stably expressing *DUOX-RNAi* were treated with soluble microbial extracts or purified microbial ligands as described in the Experimental Procedures. ROS generation was then measured using a luminal-based method, as described in the Experimental Procedures. At least five independent experiments showed similar results.

a luminal-based assay. The effects of an extract of a well-known *Drosophila*-interacting bacterium, *Erwinia carotovora carotovora-15* (*Ecc15*), which is efficiently controlled by the DUOX-dependent gut immunity, were also evaluated. The results showed that *Drosophila* S2 cells rapidly induced intracellular ROS generation upon stimulation with yeast extracts as well as upon stimulation with *Ecc15* extracts (Figure 1D). Interestingly, neither peptidoglycan nor β-1, 3-glucan induced ROS in the S2 cells, which suggests the possible involvement of other previously uncharacterized microbial pattern molecules in ROS-dependent mucosal immunity (Figure 1D). In addition, the microbe-induced ROS generation was completely abolished in S2 cells that expressed *DUOX-RNAi*, which confirmed that the observed ROS generation occurred primarily as a result of DUOX activity (Figure 1D and Figure S2). Taken together, these results indicate that *Drosophila* immunocompetent cells responded to yeast by initiating DUOX activation for ROS generation and that S2 cells might be an ideal in vitro model for elucidating intracellular signaling pathways controlling the DUOX - ROS system.

Yeast-Induced DUOX Enzymatic Activity Is Dependent on Inositol 1,4,5-Trisphosphate Generation and Subsequent Ca^{2+} Mobilization through Gαq and PLCβ Activation

We previously demonstrated that in vitro DUOX enzymatic activity from the intestinal membrane fraction is completely abolished in the absence of Ca^{2+} (Ha et al., 2005a), which suggests the involvement of intracellular Ca^{2+} in the modulation of DUOX enzymatic activity. Therefore, we tested whether yeast treatment led to the mobilization of intracellular Ca^{2+} to induce DUOX activation. *Drosophila* S2 cells were treated with yeast, and the intracellular Ca^{2+} levels were measured. The results showed that treatment with yeast increased intracellular Ca^{2+} (Figure 2A). Ca^{2+} acts as an intracellular signal to control a wide range of cellular processes, including muscle contraction, secretion, metabolism, and proliferation (Clapham, 1995). The activation of Ca^{2+} signaling depends on calcium channel activation. One of the best known mechanisms for calcium signaling is the signal-dependent release of Ca^{2+} from

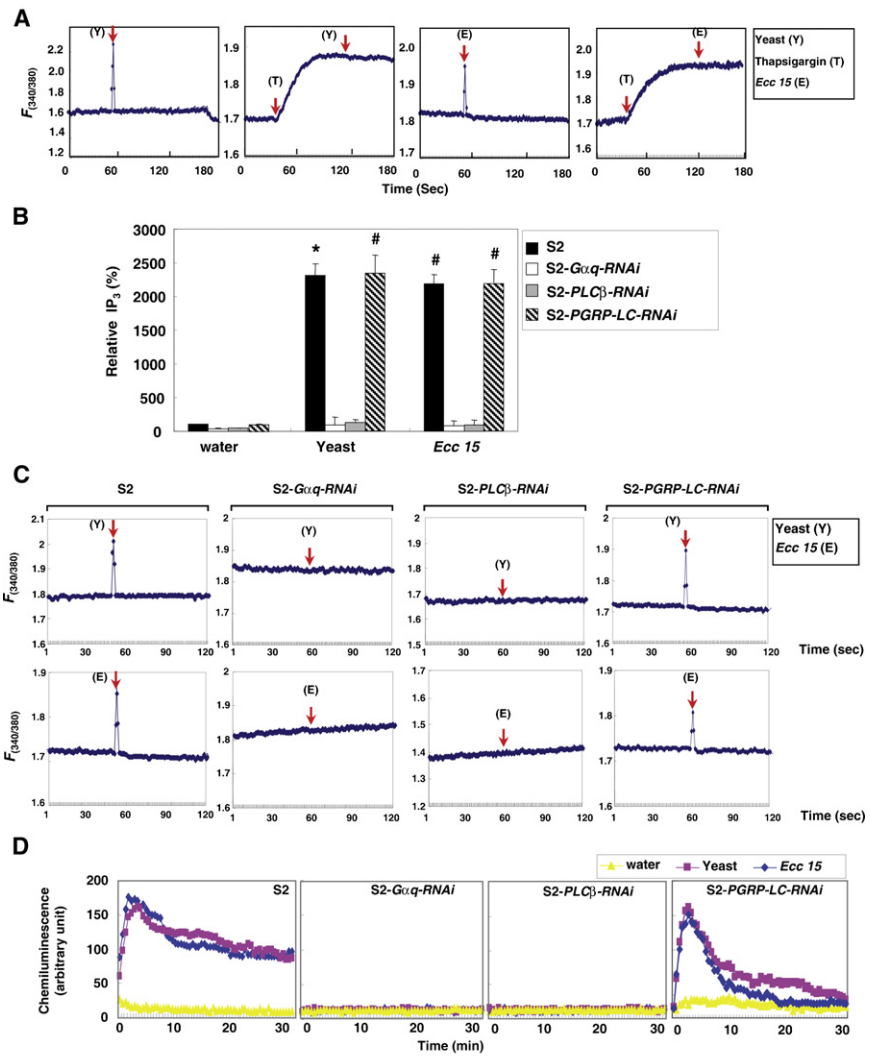

Figure 2. Yeast Induces IP_3-Dependent Ca^{2+} Mobilization through the Gαq-PLCβ Pathway for DUOX-Dependent ROS Generation

(A) Yeast induces intracellular Ca^{2+} from the endoplasmic reticulum of S2 cells. Transient fluorescence ratios (340/380) were measured in Fura-2-loaded cells in Ca^{2+}-free buffer. Soluble yeast extracts or *Ecc15* extracts were then added as described in the Experimental Procedures at a specific time point (indicated by an arrow). In the case of Ca^{2+} depletion in the endoplasmic reticulum, S2 cells were pretreated with thapsigargin (100 ng/ml) prior to stimulation with soluble microbial extracts. At least five independent experiments showed similar results.

(B–D) Yeast generates IP_3 through the Gαq-PLCβ pathway for intracellular Ca^{2+} mobilization and subsequent DUOX-dependent ROS generation. S2 cells or S2 cells stably expressing *Gαq-RNAi, PLCβ-RNAi*, or *PGRP-LC-RNAi* were treated with soluble yeast extracts or *Ecc15* extracts as described in the Experimental Procedures. IP_3 (B), Ca^{2+} (C), and ROS (D) were then measured as described in the Experimental Procedures. (B) The IP_3 basal level in the untreated cells was arbitrarily taken to be 100, and the results are presented as relative levels. Results were expressed as the mean ± SD of three different experiments. Data reflect values significantly different from the water-treated control at the $^{*}p < 0.002$ and $^{\#}p < 0.001$ (ANOVA test).

intracellular stores, such as those found in the endoplasmic reticulum (ER) (Pozzan et al., 1994). Therefore, to determine if the increase in intracellular Ca^{2+} induced by yeast was due to release from the ER stores, we pretreated S2 cells with thapsigargin to deplete the Ca^{2+} in the ER stores prior to stimulation with yeast. The results showed that thapsigargin pretreatment abolished yeast-induced Ca^{2+} mobilization (Figure 2A), indicating that yeast mobilizes Ca^{2+} from the ER stores in *Drosophila* cells. Similar results were observed when the *Ecc15* extracts were tested (Figure 2A).

Ca^{2+} released from ER stores is primarily controlled by activation of the inositol 1,4,5-trisphosphate (IP_3) receptor in the ER membrane (Berridge, 1993), which can be generated from the hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, by phospholipase C-β (PLCβ) during signal transduction (Rhee, 2001). PLCβ is activated by the GTP-bound subunit of the Gq class of heterodimeric G proteins (Jhon et al., 1993; Lee et al., 1994; Rhee, 2001). Therefore, to determine if yeast induced the generation of IP_3 in a Gαq- and PLCβ-dependent manner, the IP_3 concentration was measured following yeast treatment. The results showed that yeast treatment induced the generation of IP_3 in S2 cells (Figure 2B) and that this yeast-induced IP_3 generation was abolished in S2 cells expressing either *Gαq-RNAi* or *PLCβ-RNAi* (Figure 2B and Figure S2). Consistent with these results, the yeast-induced Ca^{2+} release and subsequent ROS generation were completely abolished in S2 cells that expressed either *Gαq-RNAi* or *PLCβ-RNAi* (Figures 2C and 2D). Similar results were observed with *Ecc15* treatments (Figures 2B–2D). Microbe-induced IP_3 generation, Ca^{2+} release, and subsequent ROS generation, however, were normal in the absence of IMD-pathway potential (i.e., in the absence of the essential receptor for IMD pathway, peptidoglycan recognition protein [PGRP]-LC [Choe et al., 2002; Kaneko et al., 2006; Werner et al., 2003]) (Figures 2B–2D and Figure S2), although microbe-induced AMP expression was severely impaired under the same condition (Figure S3). This finding indicated that the peptidoglycan-mediated IMD pathway is not involved in DUOX-dependent ROS generation. Taken together, these results indicated that yeast activates PLCβ, which in turn generates IP_3, leading to a release of Ca^{2+} from the ER stores. In addition, Gαq- and PLCβ-dependent Ca^{2+} mobilization is an essential step for the induction of DUOX enzymatic activity and subsequent microbicidal ROS generation.

In Vivo Gut-Yeast Interaction Mobilizes Ca^{2+} for ROS Generation through Dynamic Activation of Gαq and PLCβ

The above in vitro data suggest that the continuous gut-yeast interactions that occur in a natural environment may dynamically activate PLCβ to mobilize Ca^{2+} to induce the DUOX-dependent ROS system in the gut epithelia. Therefore, to determine if microbe-induced Ca^{2+} mobilization occurred during the in vivo gut-yeast interaction, intracellular Ca^{2+} induction was examined using fluorescence resonance energy transfer (FRET) analyses (Fiala and Spall, 2003; Jares-Erijman and Jovin, 2003). To accomplish this, we used transgenic flies carrying a genetically engineered fluorescence sensor, cameleon protein (Fiala and Spall, 2003). Cameleon protein consists of an enhanced cyan fluorescence protein (ECFP) as an energy donor and an enhanced yellow fluorescence protein (EYFP) as an energy acceptor (Miyawaki et al., 1999, 1997). Both fluorescence proteins in cameleon are fused by a linker domain containing the calcium-binding region of calmodulin and the calmodulin target peptide, M13, which results in its being Ca^{2+} responsive (Figure 3A) (Miyawaki et al., 1999, 1997). FRET analysis was performed using an acceptor photobleaching method (Bastiaens et al., 1996; Daniels et al., 2004), and the CFP signals of the calcium-bound form of cameleon protein were then enhanced after YFP was bleached in the regions of interest (Figure 3A). The result revealed that Ca^{2+} induction was observed in the proventriculus and in the upper and lower regions of iron and copper cells of the posterior midgut of the cameleon flies in the presence of yeast, confirming that yeast can mobilize intracellular Ca^{2+} in vivo (Figures 3B and 3C).

The involvement of PLCβ in the induction of Ca^{2+} during the in vivo gut-yeast interaction was then evaluated based on PLCβ expression in the gut. Two subtypes of PLCβ (subtype-I and -II) are produced by alternative splicing of *norpA* gene transcripts (Kim et al., 1995). *PLCβ/norpA* subtype-I is exclusively expressed in the retina and is required for phototransduction in the eye (Kim et al., 1995). Using isoform-specific primers, we observed that *PLCβ* subtype-II, but not *PLCβ* subtype-I, is expressed in the gut epithelia (Figure 4A), indicating that *PLCβ* subtype-II is the dominant *norpA* transcript form in the gut. Next, we generated transgenic flies that expressed PLCβ subtype-II fused to red fluorescence protein (RFP) to monitor in vivo PLCβ activation in the gut. Activated PLCβ is known to be localized in the membrane region of the cells (Rhee, 2001). The results showed that PLCβ is predominantly cytoplasmic in the gut cells in the absence of yeast (using flies fed on sucrose), whereas it is localized in the membrane of the gut cells in the presence of yeast (using flies fed on the live yeast media) (Figure 4B). Consistently, treatment of yeast extract could also induce rapid membrane localization of the *PLCβ*-transfected S2 cells (Figure 4C). However, yeast-induced PLCβ activation was abolished in the gut of *Gαq* mutant flies, demonstrating that Gαq acts as an upstream signaling component for PLCβ activation (Figure 4B). This result indicated that the Gαq-PLCβ pathway is dynamically activated in the presence of nutritional microbes in the gut epithelia.

We then evaluated the Ca^{2+} activation state using acceptor photobleaching FRET analyses in the presence or absence of the Gαq-PLCβ pathway to determine if the Gαq-PLCβ pathway

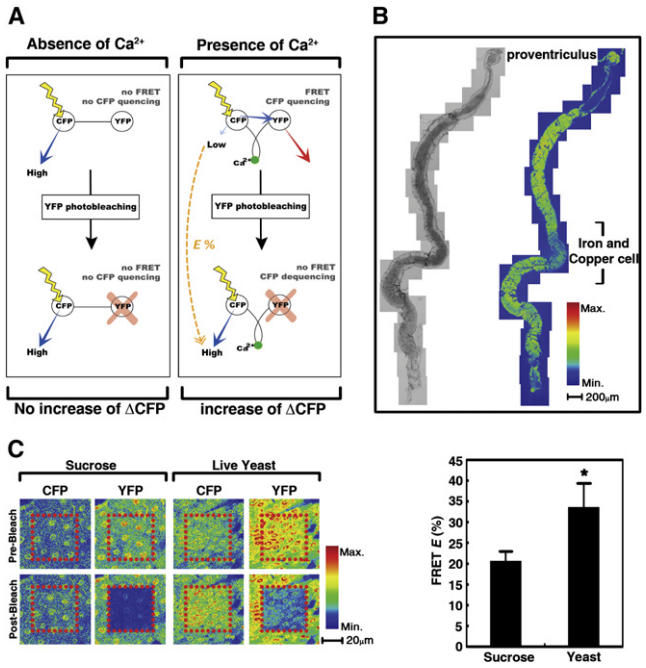

Figure 3. Yeast Induces Intracellular Ca^{2+} in the Gut Epithelia

(A) Schematic presentation of the Ca^{2+} analysis of the cameleon calcium sensor protein by the acceptor photobleaching method. In the presence of Ca^{2+}, CFP (donor) and YFP (acceptor) were located in close proximity, which resulted in donor quenching (little fluorescence being emitted from the CFP) due to the FRET from CFP to YFP. Following depletion of the YFP by the acceptor photobleaching methods in the region of interest, donor dequenching (increased CFP emission) in the region of interest could be observed due to the absence of FRET. FRET efficiency (*E*%) was quantified by assessing donor dequenching after acceptor photobleaching as described previously (Jobin et al., 2003). Consequently, *E*% assessed by the change in CFP emission upon photobleaching increases in the region of interest in the presence of Ca^{2+}.

(B and C) Yeast induces in vivo Ca^{2+} in the gut. Flies (3 to 5 days old) maintained in either live yeast media or sucrose alone for 24 hr were analyzed. Whole gut (B) and upper region of iron and copper cells (C), morphologically large cells known to accumulate copper, were analyzed by the acceptor photobleaching method. A pseudocolored FRET image showed the ratio of donor (CFP) emissions after YFP photobleaching (B) and before/after YFP photobleaching (C). (C) The region of interest is indicated by the red box. Representative FRET images are shown in the left panel. FRET efficiency (*E*%) was also quantified as described in Figure 3A and is expressed as the mean ± SD of 50 different regions of interest from 50 flies (right panel). Asterisk reflects value significantly different from the sucrose control at the $p < 0.016$ (ANOVA test).

is necessary for intracellular in vivo Ca^{2+} mobilization. The results showed that yeast-induced Ca^{2+} mobilization was greatly abolished in the gut of *Gαq-RNAi* flies (Figure 5A and Figure S4). A similar result was observed in the gut of *PLCβ* mutant flies (Figure 5B). Consistent with this result, the in vivo gut ROS level was markedly decreased in the absence of either Gαq or PLCβ when compared to that of control flies (Figure 5C). In addition, the impaired Ca^{2+} mobilization and the low ROS level in the *PLCβ* mutant flies was completely restored by genetic reintroduction of PLCβ-RFP expression (Figures 5B and 5C). In these PLCβ-rescued flies, intracellular Ca^{2+} was observed in cells containing membrane-targeted activated PLCβ (Figure 5B). Taken together, these results demonstrate that DUOX-dependent

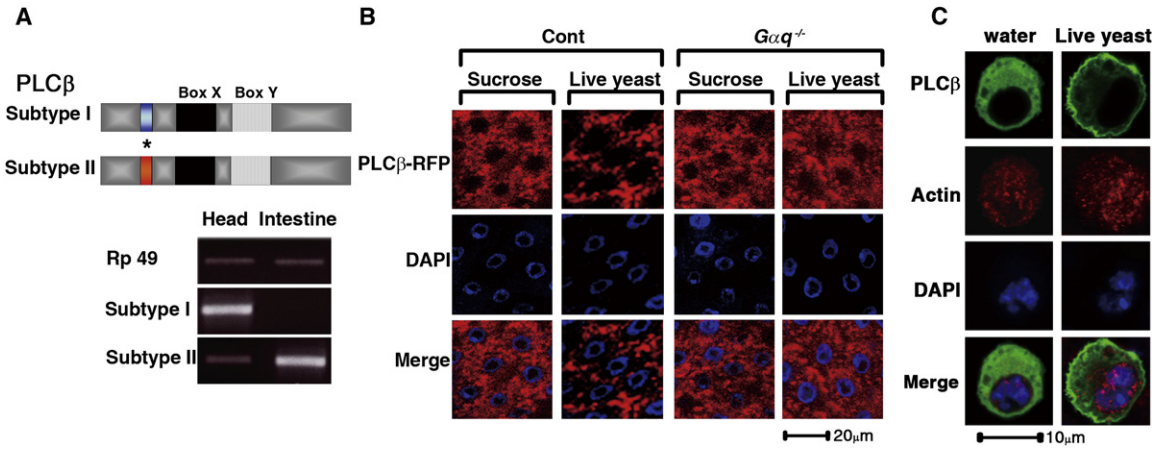

Figure 4. *PLCβ/norpA* Subtype-II Is Expressed and Activated in the Gut in a Gαq-Dependent Manner

(A) Expression of *PLCβ/norpA* subtype-II in the gut. Schematic representation of PLCβ splice-variant subtype-I and -II (upper panel). The highly conserved Box X and Box Y regions (Rhee and Choi, 1992) that are present in the major type of PLC are indicated. The alternatively spliced region is indicated by an asterisk. RT-PCR analysis of PLCβ subtype expression in the gut epithelia and head (lower panel).

(B) Activation of PLCβ subtype-II can be observed in the gut in a Gαq-dependent manner. Transgenic flies carrying PLCβ-RFP (*UAS-PLCβ-RFP/Da-GAL4*) were used to monitor the membrane-targeted activated form of PLCβ. Flies (3 to 5 days old) maintained in either live yeast media or sucrose alone for 24 hr were analyzed. No PLCβ activation was observed in the gut of *Gαq* mutant flies (*Gaq^1/ Gaq^1; UAS-PLCβ-RFP/Da-GAL4*). Nuclear staining was performed with DAPI (blue).

(C) Yeast treatment induces the membrane localization of the activated form of PLCβ subtype-II. V5-epitope-tagged PLCβ subtype-II was transfected into the S2 cells. PLCβ (green) and actin (red) was localized after 10 min of water or yeast extract treatment.

intestinal ROS generation is dependent on the Gαq-PLCβ-Ca^{2+} pathway, both in vivo and in vitro.

The Gαq-PLCβ-DUOX Pathway Is Required for Routine Control of Nutritional Microbes and Host Survival

Because the interaction with yeast is continuous during the *Drosophila* life cycle, the above results suggest that dynamic activation of the Gαq-PLCβ pathway is important for the routine control of yeast and to achieve proper host nutrition and survival. Therefore, we examined the survival rate of *Gαq* and *PLCβ* mutant flies fed on the live yeast media to examine the role that Gαq-PLCβ plays in the maintenance of gut homeostasis and host health in a normal yeast-rich gut environment. The results showed that the survival rates of *Gαq* and *PLCβ* mutant flies were significantly lower than those of control flies (Figure 6C), similar to *DUOX-RNAi* flies (Figures S4 and S5). As feeding rates of mutant flies were similar to those of control flies regardless of food types (i.e., live yeast or dead yeast media) (Figure S6), it is likely that alteration of feeding rate is not involved in the host mortality of the mutant flies. Consistent with this result, tissue-specific introduction of either *Gαq-RNAi* or *PLCβ-RNAi* in the gut, but not in the fat body/hemocytes, resulted in host mortality (Figures S4 and S7), demonstrating the importance of intestinal Gαq-PLCβ-DUOX signaling in host survival. To determine if the low survival rates exhibited by the *Gαq* or *PLCβ* mutant flies were the result of an inability to control the live yeast, we evaluated the CFUs of yeast in the gut. The results showed that the CFUs of yeast in the gut were more than 100 times greater in *Gαq* and *PLCβ* mutant flies than in control flies (Figure 6A). In addition, gut pathology analyses revealed that the gut cells of *Gαq* and *PLCβ* mutant flies in the live yeast media, but not in the dead yeast media, showed severe apoptosis (Figure 6B), which suggests that uncontrolled proliferation of yeast may induce gut cell apoptosis and ultimately host death. Consistent with these findings, the lethality of *Gαq* and *PLCβ* mutant flies was significantly ameliorated when they were maintained in the dead yeast media (Figure 6C). It is not clear how exactly yeast induces gut cell apoptosis of Gαq-PLCβ-DUOX pathway mutant flies. One possibility is that uncontrolled high numbers of local yeast in the gut of these mutant flies may produce toxic metabolites that may lead to gut apoptosis and ultimately host death. It is also possible that these mutant flies starved to death as they could not digest live yeast. To test the latter possibility, the flies were maintained in the mixed media containing both live and dead yeast to avoid the starvation effect. In this condition, we observed that feeding rates of mutant flies were similar regardless of food types (Figure S6). The result showed that the high lethality was still observed in all of tested mutants (*Gαq*, *PLCβ*, and *DUOX-RNAi*) (Figure S8), indicating that the major cause of death is not the starvation effect. Furthermore, the survival rates of mutant flies which were normal when fed with dead yeast media were shown to be affected by gut infection with different microbes (Figure S9) but not by systemic infection (Figure S10). These results indicated that the Gαq-PLCβ pathway is involved in gut immunity but not in systemic immunity. It is important to note that when *PLCβ* was reintroduced into the gut of *PLCβ* mutant flies to rescue the impaired Ca^{2+} signaling, the low microbe-eliminating capacity, the high gut apoptosis rate, and the low survival rates of the *PLCβ* mutant flies were almost completely restored to those of the control flies (Figures 6A–6C). Furthermore, the high lethality of the partial loss-of-function mutant of *PLCβ* (*PLCβ-RNAi* flies), but not that of null mutant of *PLCβ* (*$norpA^7$*), could be rescued by ectopic overexpression of *DUOX* (Figure S11). This result indicates that

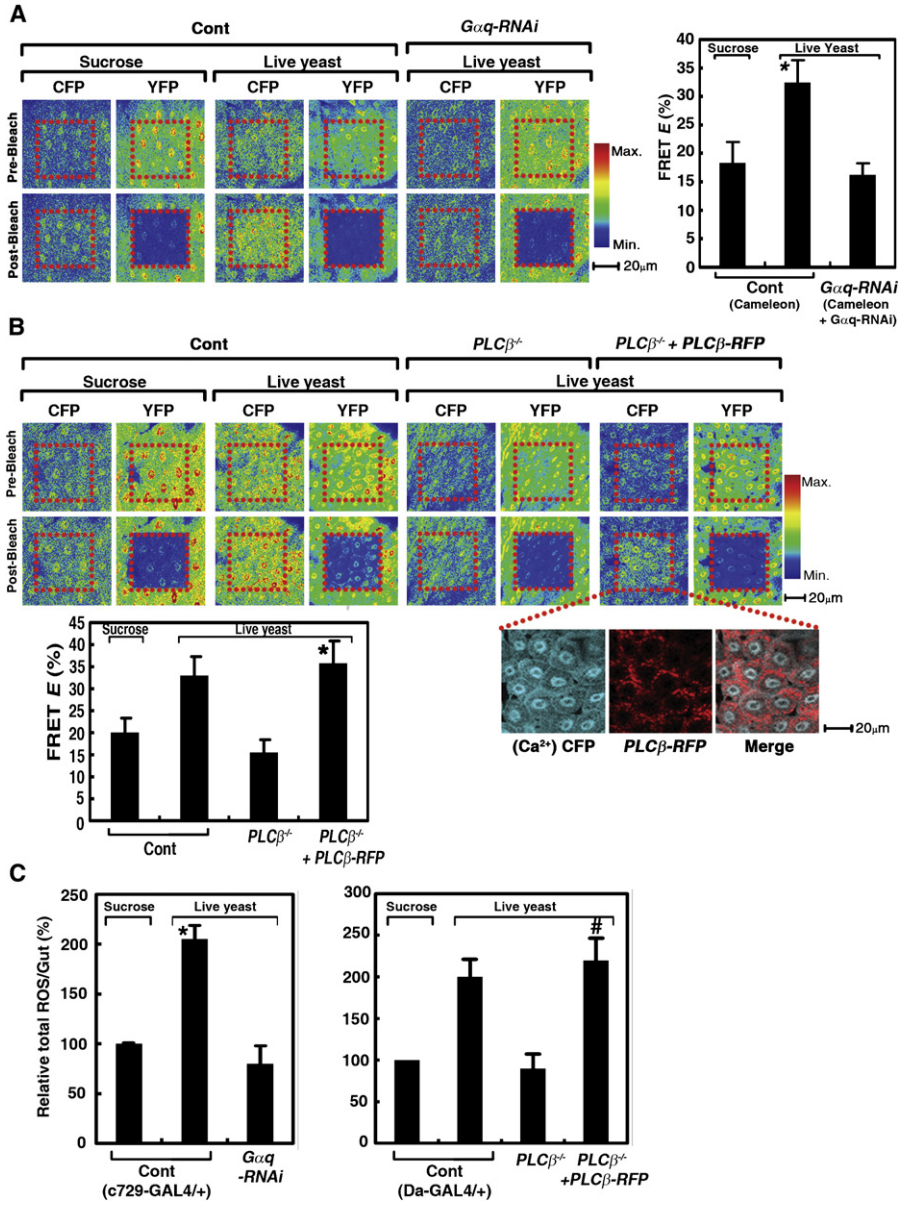

Figure 5. In Vivo Ca^{2+} Mobilization and ROS Generation in the Gut Are Dependent on the Gαq-PLCβ Pathway

(A and B) In vivo Ca^{2+} mobilization is mediated by Gαq and PLCβ. Flies (3 to 5 days old) maintained in either live yeast media or sucrose alone for 24 hr were analyzed. *Gαq* mutant (A) and *PLCβ* mutant (B) were analyzed by the acceptor photobleaching method. A pseudocolored FRET image shows the ratio of donor (CFP) emissions before and after YFP photobleaching. The region of interest is indicated by the red box. Representative FRET images are shown. FRET efficiency (*E*%) was also quantified as described in Figure 3A and is expressed as the mean ± SD of 50 different regions of interest from 50 flies. Asterisk reflects value significantly different from the sucrose control at the $p < 0.02$ (ANOVA test). The genotypes of the flies used in Figure 5A were as follows: Control (*UAS-cameleon-2.1/c729-GAL4*) and Gαq-RNAi (*UAS-cameleon-2.1/c729-GAL4; UAS-Gαq-RNAi*/+). The genotypes of the flies used in Figure 5B were as follows: Control (*UAS-cameleon-2.1/+; Da-GAL4*/+); PLCβ$^{-/-}$ (*norpA*^{7}*; UAS-cameleon-2.1/+; Da-GAL4*/+); and PLCβ$^{-/-}$ + PLCβ (*norpA*^{7}*; UAS-cameleon-2.1/+; Da-GAL4/ UAS-PLCβ-RFP)*. In the PLCβ$^{-/-}$ + PLCβ flies, activated PLCβ-RFP (red) was colocalized in Ca^{2+}-expressing cells (cyan) after YFP-photobleaching. Asterisk reflects value significantly different from the *PLCβ*$^{-/-}$ flies fed on live yeast at the $p < 0.001$ (ANOVA test).

(C) Basal in vivo ROS level in the gut epithelia. Flies (3 to 5 days old) maintained in either live yeast media or sucrose alone for 24 hr were analyzed. The genotypes of flies used in this study were as follows: Da-GAL4 (*Da-GAL4*/+); c729-GAL4 (*c729-GAL4*/+); Gαq-RNAi (*c729-GAL4/+; UAS-Gαq-RNAi*/+); PLCβ$^{-/-}$ (*norpA*^{7}*; Da-GAL4*/+); and PLCβ$^{-/-}$ + PLCβ (*norpA*^{7}*; Da-GAL4/ UAS-PLCβ-RFP*). The ROS level in the control intestine was arbitrarily taken to be 100, and the results are presented as relative levels. Results are expressed as the mean ± SD of three different experiments. ANOVA test showed that values are significantly different from the control flies fed on sucrose (*$p < 0.001$) and from the *PLCβ*$^{-/-}$ flies fed on live yeast (#$p < 0.001$).

low Ca^{2+} signaling, but not complete absence of Ca^{2+} signaling, can be overcome by increasing *DUOX* level. This result further indicates that the Ca^{2+} signaling, which is absolutely required for the mucosal immunity, acts as an upstream event for DUOX activity. Consistent with these results, the low survival rate of *DUOX-RNAi* flies was rescued by the gut-specific

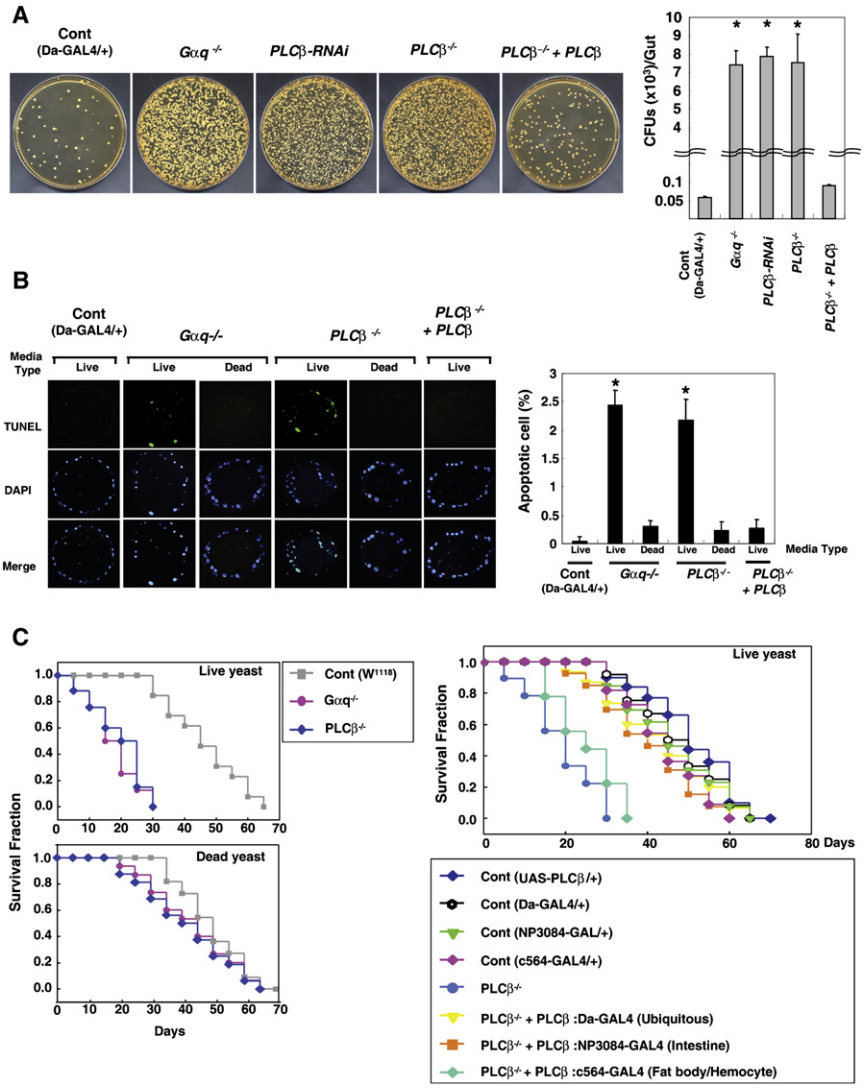

Figure 6. The Gαq-PLCβ-Ca^{2+}-DUOX Pathway Is Required for Routine Microbial Control, Gut Homeostasis, and Normal Host Survival

The genotypes of flies used in this study were as follows: Control (*Da-GAL4/+*); $G\alpha q^{-/-}$ (*Gaq*^{1}); PLCβ-RNAi (*UAS-PLCβ-RNAi/+; Da-GAL4/+*); $PLC\beta^{-/-}$ (*norpA*^{7}); and $PLC\beta^{-/-}$ + PLCβ (*norpA*^{7}*; Da-GAL4/UAS-PLCβ-RFP*).

(A) The Gαq-PLCβ pathway is required for routine control of dietary yeast in the gut. Yeast persistence was measured as described in Figure 1B. Representative plates of yeast recovered from the intestines are shown in the left panel. The number of CFUs per adult intestine (right panel) represents the mean ± SD of three different experiments. Asterisks reflect values significantly different from the control at the $p < 0.001$ (ANOVA test).

(B) The Gαq-PLCβ pathway is required for gut homeostasis in the presence of dietary microbes. Flies were maintained under live yeast or dead yeast media for 10 days before analyses. The apoptosis index of the posterior midgut cells was determined by dividing the number of apoptotic cells by the total number of cells and multiplying by 100. Values represent the means ± SD of five independent experiments. Asterisks reflect values significantly different from the control flies fed on live yeast at the $p < 0.001$ (ANOVA test). Apoptosis index (right panel) and representative images of TUNEL staining (left panel). TUNEL-positive cells are green.

(C) The Gαq-PLCβ-Ca^{2+}-DUOX pathway is required for host survival during routine gut-microbe interactions. Survival rates were assessed under live yeast or dead yeast media without infection. In all cases, survival in three or more independent cohorts of approximately 25 flies each was monitored over time. Log rank analysis showed statistically significant differences in survival in live yeast media between w^{1118} control flies and *PLCβ* (or *Gαq*) mutant flies ($p < 0.0001$), between *norpA*^{7} and *norpA*^{7}*; NP3084-GAL4/+; UAS-PLCβ-RFP/+* ($p < 0.0006$), and between *norpA*^{7} and *norpA*^{7}*; UAS-PLCβ-RFP/Da-GAL4* ($p < 0.0001$). In a tissue-specific rescue experiment (right panel), the *NP3084-GAL4* driver (*norpA*^{7}*; NP3084-GAL4/+; UAS-PLCβ-RFP/+*), *c564-GAL4* drivers (*norpA*^{7}*; c564-GAL4/+; UAS-PLCβ-RFP/+*); and *Da-GAL4* driver (*norpA*^{7}*; UAS-PLCβ-RFP/Da-GAL4*) were used for intestine-specific, fat body/hemocyte-specific, and ubiquitous *PLCβ* expression, respectively. The w^{1118}, *GAL4* only, and *UAS* only flies were used as controls.

reintroduction of full-length *DUOX* but not by the Ca^{2+}-insensitive form of *DUOX* lacking Ca^{2+}-modulated EF-hand domains (Nakayama and Kretsinger, 1994) (Figure S5). Taken together, these results indicate that the Gαq-PLCβ-Ca^{2+}-dependent DUOX-activation pathway is required for the routine control of dietary yeast, which is essential for gut homeostasis and host nutrition.

DISCUSSION

Different tissues within an organism must develop specific microbial-controlling systems to maintain life. Most internal tissues normally function under aseptic conditions and encounter microbes only when they are accidentally introduced from outside the body. In *Drosophila*, these invading microbes are then eliminated by the systemic immune system, primarily via NF-κB pathway-dependent AMP production in fat body cells (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). The gut epithelia, however, generally act as a physical barrier to prevent microbial invasion; therefore, they exist under septic conditions. This suggests the existence of a signaling pathway that regulates the constant contact with microorganisms. Several lines of evidence suggest that gut epithelia generate ROS (El Hassani et al., 2005; Geiszt et al., 2003b; Ha et al., 2005a), which may then provide an essential mucosal surface host defense mechanism. The NADPH oxidase family, notably NOX and DUOX, are primarily involved in the generation of ROS by various nonphagocytic cells, including those of the gut epithelia (Lambeth, 2004). Although the involvement of specific NOX and/or DUOX in gut immunity has not yet been demonstrated in vertebrate animals,

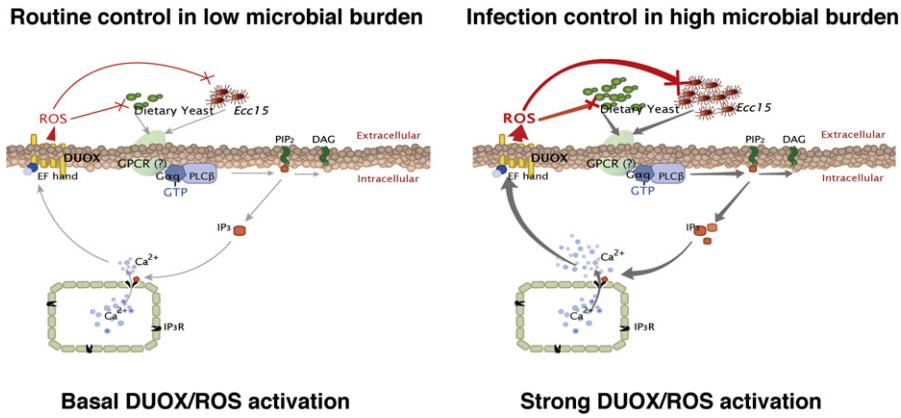

Figure 7. Model for DUOX-Activating Signaling Pathway

Under routine and trivial microbial contacts (e.g., during naturally occurring gut-yeast interactions), a microbe-derived ligand (or the stress caused by different microbial burden) may activate G protein-coupled receptor (GPCR) and/or G protein by replacing GDP with GTP in the Gαq subunit through an unknown mechanism. The freed Gαq may activate PLCβ, which hydrolyzes a minor phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 is released from the membrane and binds to the IP_3 receptor (IP_3R) located in the ER membrane, a process which appears to be required in intracellular calcium induction. Subsequently, released Ca^{2+} may positively modulate DUOX activity through a Ca^{2+}-sensitive EF hand domain of the DUOX to produce microbicidal ROS. In the condition of high bacterial burden (e.g., during oral infection with a pathogen such as *Ecc15*), DUOX signaling pathway would be strongly activated for the ROS-dependent pathogen elimination.

DUOX has been shown to be involved in microbe-induced in vivo ROS generation and host defense in the gut of *Drosophila* (Ha et al., 2005a). Thus, host redox response to microorganisms is not restricted to phagocytes but is also used in other cells such as mucosal barrier epithelia. Several studies have suggested that bacterial-induced ROS generation in the gut epithelial cells may modulate diverse signaling pathways (Lee, 2008), thereby actively shaping host physiology. Dysregulation of ROS generation and/or the ROS elimination system seems to be deeply involved in many chronic inflammatory diseases frequently found in the microbe-contacting gut epithelia (Geiszt et al., 2003a; Geiszt and Leto, 2004), which suggests the importance of redox regulation during microbe-gut interaction. However, our understanding of the intestinal redox system is limited, primarily due to a lack of knowledge regarding the signaling pathway(s) by which gut-microbe interactions initiate ROS generation. In this study, we demonstrated that the Gαq-PLCβ-Ca^{2+} signaling pathway controls the mucosal gut epithelial defense system through DUOX-dependent ROS generation, which is responsible for routine microbial interactions in the gut epithelia in the absence of infection (Figure 7). The PLCβ pathway impacts a wide variety of biological processes through the generation of a lipid-derived second messenger (Rhee, 2001). In this process, the hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, by PLCβ generates two intracellular messengers, IP_3 and diacylglycerol (Rhee, 2001). This process is one of the earliest events through which more than 100 extracellular signaling molecules regulate functions in their target cells. Previously, it was shown that Gαq-PLCβ signaling is essential for the activation of the phototransduction cascade in *Drosophila* (Bloomquist et al., 1988; McKay et al., 1995; Schneuwly et al., 1991; Scott et al., 1995). Our study revealed a physiological role of PLCβ wherein it is involved in the regulation of DUOX enzymatic activity, which leads to the generation of microbicidal ROS in the mucosal epithelia.

PLCβ signaling is very rapid, with only a few seconds necessary to activate Ca^{2+} release and ROS production. This extremely rapid response may be advantageous for the host and may be the mechanism by which dynamic and routine control of microbes in the gut epithelia is achieved. Because the gut is in continuous contact with microbes such as dietary microorganisms, it is conceivable that under normal conditions routine microbial contact dynamically induces a certain level of basal Gαq-PLCβ activity that varies depending on the local microbe concentration. This basal Gαq-PLCβ-DUOX activity seems to be sufficient for host survival (Figure 7). In such conditions of low bacterial burden, NF-κB-dependent AMP expression is known to be largely repressed by Caudal repressor for the preservation of commensal microbiota (Ryu et al., 2008). However, in the case of high bacterial burden (e.g., gut infection condition), the DUOX-ROS system would be strongly activated for full microbicidal activity (Figure 7). Furthermore, all of the flies that contained impaired signaling potentials for the Gαq-PLCβ-Ca^{2+}-DUOX pathway were totally intact following septic injury (Figure S10) but short-lived under natural rearing conditions (Figure 6 and Figure S7) or under gut infection conditions (Figure S9), indicating that the mucosal immune pathway is distinct from the systemic immune pathway.

It is not clear how Gαq- and PLCβ-induced Ca^{2+} modulates DUOX enzymatic activity. Because the DUOX lacking Ca^{2+}-binding EF hand domains is unable to rescue the *DUOX-RNAi* flies (Figure S5) and because ROS-producing DUOX enzymatic activity is directly dependent on intracellular Ca^{2+} (Ha et al., 2005a), it is plausible that Ca^{2+} directly modulates the enzymatic activity of DUOX through binding to the EF hand domains.

It is also important to determine what pathogen-associated molecular patterns (PAMPs) are responsible for the activation of PLCβ signaling. In *Drosophila*, peptidoglycan and β-1,3-glucan are the only two PAMPs known to induce the NF-κB signaling pathway in the systemic immunity (Gottar et al.,

2006; Leulier et al., 2003). Our results showed that neither peptidoglycan nor β-1,3-glucan was able to induce ROS in S2 cells, which suggests that a previously uncharacterized type(s) of PAMP is involved in the mucosal immunity. Because the Gαq protein acts as an upstream signaling component of the PLCβ-Ca^{2+} pathway, a microbe-derived ligand capable of activating G protein coupled receptor(s) and/or Gαq protein may be the best candidate for the Gαq-PLCβ-Ca^{2+}-DUOX signaling pathway. Given the broad spectrum of microbes that activate the response, it remains possible that the unknown upstream sensors resemble a stress response more than a PAMP response. Elucidation of the molecular nature of such agonists will greatly enhance our understanding of bacteria-modulated redox signaling in the gut epithelia. In conclusion, this study demonstrates that mucosal epithelia have evolved an innate immune strategy, which is functionally distinct from the NF-κB-dependent systemic innate immune system. The rapid Gαq-PLCβ-Ca^{2+}-DUOX signaling is adapted to the routine and dynamic control of gut-associated microbes and may impact the long-term physiology of the intestine and host fitness.

EXPERIMENTAL PROCEDURES

Constructs and Fly Strains

pUAST vectors containing different DNA constructs (*UAS-Gαq-RNAi, UAS-PLCβ-RNAi*, and *UAS-PLCβ-RFP*) were used to generate transgenic animals via P element-mediated transformation (Rubin and Spradling, 1982). These constructs were then microinjected into w^{1118}-expressing embryos.

Flies were maintained on standard cornmeal-agar medium at 25°C with 60% relative humidity. The following fly lines, which have been described previously, were also used in this study: *UAS-cameleon2.1* (Fiala and Spall, 2003); *UAS-DUOX-RNAi* (Ha et al., 2005a); *UAS-DUOX* (Ha et al., 2005a); *UAS-DUOX-ΔEF* (Ha et al., 2005a); $Gαq^1$ (Scott et al., 1995); $norpA^7$ (McKay et al., 1995); Dif^1 (Meng et al., 1999); Rel^{E20}(Hedengren et al., 1999); *NP3084-GAL4* (Nehme et al., 2007); *c564-GAL4* (Harrison et al., 1995); *c729-GAL4* (Petersen et al., 1999); and *Da-GAL4* (Giebel et al., 1997). In this study, genetic backgrounds of $Gαq^1$ and $norpA^7$ were not taken into account. To avoid potential problems stemming from the different genetic backgrounds, *PLCβ−RNAi* and *Gαq-RNAi* flies were also tested with different *GAL4* drivers.

ROS Measurement

S2 cells (~5 × 10^5) were harvested and washed with HEPES-buffered saline (120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 17 mM HEPES [pH 7.4]). The superoxide-producing activity was then determined using superoxide dismutase-inhibitable chemiluminescence with an enhancer-containing luminal-based detection system (Diogenes, National Diagnostics, E. Yorkshire, UK), as described previously (Choi et al., 2008). Following the addition of the enhanced luminal-based substrate, the cells were stimulated with various ligands (soluble microbial extract [25 μg/ml], peptidoglycan [5 μg/ml], or β-1,3-glucan [5 μg/ml]), and chemiluminescence was measured using a luminometer (Microlumat Plus LB96V, Berthold Technologies, Bad Wildbad, Germany). The in vivo ROS in the dissected intestine were measured using a ferric-xylenol orange assay, as described previously (Ha et al., 2005a).

Calcium Measurement

Intracellular Ca^{2+} was measured using a previously described method (Rosker et al., 2004). In brief, S2 cells (10^8 cells) were loaded with 2 mM Fura-2-acetoxymethyl ester for 40 min at room temperature in a buffer containing 10 mM glucose, 140 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM HEPES, at pH 7.4. The cells were then washed twice with a Ca^{2+} chelating buffer containing 10 mM glucose, 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM KCl, 1 mM $MgCl_2$ and 10 mM HEPES, at pH 7.4, and then resuspended in the same buffer. Soluble microbial extract (10 μg per 10^6 cells) was added to the cells. The fluorescence was monitored with a fluorescent spectrophotometer (Shimadzu, Japan) by continuous recording at 0.5 s intervals at room temperature (excitation at 340 and 380 nm and emission at 510 nm).

IP_3 Measurement

S2 cells (10^7 cells) were treated with soluble microbial extracts (25 μg/ml) for 10 min. Cells were then homogenized in an ice-cold trichloroacetic acid (7% weight per volume). The trichloroacetic acid-soluble supernatants were extracted with diethyl ether and neutralized with KOH. IP_3 contents in the supernatants were determined using a Radioreceptor Assay Kit (Perkin Elmer) according to the manufacturer's instructions. Pellets were dissolved in 0.1 N NaOH, and protein concentrations were determined using the Bradford protein assay. A standard curve was established with a known amount of IP_3 (0–120 pmol/ml) according to the manufacturer's instructions.

FRET Analysis

The dissected guts of flies (3 to 5 days old) expressing cameleon calcium sensor were fixed and plated onto coverslips for FRET analysis (Fiala and Spall, 2003; Jares-Erijman and Jovin, 2003). FRET images were obtained using a LSM510 Meta Confocal Microscope (Carl Zeiss, Germany). FRET analysis by the acceptor bleaching method was performed as described previously (Bastiaens et al., 1996; Daniels et al., 2004; Jobin et al., 2003).

Survival Experiments

Flies with different genotypes were reared on dead yeast media (composed of autoclaved cornmeal agar media supplemented with 2% heat-killed yeast) up to the early adult stage at day 2. Adult flies (2 days old) were transferred into a fresh vial containing live yeast media (autoclaved cornmeal agar media supplemented with 2% live yeast) or dead yeast media for survival experiments. Dried live *S. cerevisiae* was purchased from Lesaffre Yeast Corp. Flies were then transferred into a fresh vial every 4 or 5 days.

For the septic infection treatment, flies (3 or 4 days old) were pricked with a fine needle that had been dipped into a concentrated culture of microbes (OD at 600 nm = 100 for yeast and *Ecc15*, and OD at 600 nm = 50 for *E. faecalis*) as described previously (Jang et al., 2006). All animals were maintained in dead yeast media at 25°C. In all cases, survival in three or more independent cohorts comprising approximately 25 flies each was monitored over time.

SUPPLEMENTAL DATA

Supplemental Data include eleven figures and Supplemental Experimental Procedures and can be found with this article online at http://www.developmentalcell.com/supplemental/S1534-5807(09)00029-X.

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