Innate Immune Homeostasis by the Homeobox Gene Caudal and Commensal-Gut Mutualism in Drosophila

Ji-Hwan Ryu,1* Sung-Hee Kim,1* Hyo-Young Lee,1,2 Jin Young Bai,1 Young-Do Nam,3 Jin-Woo Bae,3 Dong Gun Lee,4 Seung Chul Shin,1,5 Eun-Mi Ha,1 Won-Jae Lee1†

Although commensalism with gut microbiota exists in all metazoans, the host factors that maintain this homeostatic relationship remain largely unknown. We show that the intestinal homeobox gene Caudal regulates the commensal-gut mutualism by repressing nuclear factor kappa B–dependent antimicrobial peptide genes. Inhibition of Caudal expression in flies via RNA interference led to overexpression of antimicrobial peptides, which in turn altered the commensal population within the intestine. In particular, the dominance of one gut microbe, Gluconobacter sp. strain EW707, eventually led to gut cell apoptosis and host mortality. However, restoration of a healthy microbiota community and normal host survival in the Caudal-RNAi flies was achieved by reintroduction of the Caudal gene. These results reveal that a specific genetic deficiency within a host can profoundly influence the gut commensal microbial community and host physiology.

The mucosal epithelia of all metazoans, such as those found in the gastrointestinal tract, are in intimate contact with a large number of commensal microbiota (1). As a consequence, commensal bacteria are known to influence many aspects of the host gut physiology, including innate immunity, development, and homeostasis (2–7). However, the lack of a genetically amenable animal model has limited in-depth analyses of gut-microbe interactions in vivo.

Recent studies have shown that the Drosophila gut activates host antimicrobial defense through the production of microbialicidal reactive oxygen species (ROS) and antimicrobial peptides (AMPs) (8–11). During most gut-pathogen interactions, intestinal redox homeostasis, mediated via infection-induced ROS generation by the dual oxidase enzyme and subsequent ROS elimination by immune-regulated catalase, is critical for host survival (8, 9). The direct contact between gut epithelia and ingested pathogens also activates the immune deficiency (IMD) pathway and subsequent nuclear localization of the p105-like NF-κB, Relish, which in turn leads to de novo synthesis of diverse immune effectors: AMPs and immunosuppressive enzymes such as the peptidoglycan recognition proteins PGRP-SC and PGRP-LB (10, 12–16). Although pathogen-initiated gut immunity is fairly well documented in Drosophila (8–10, 14, 16, 17), the molecular interaction between commensals and the Drosophila gut is poorly understood.

Role of gut commensal microbiota in the IMD-Relish pathway. To investigate the molecular mechanism for commensal-gut interactions, we first asked whether the commensal microbiota could elicit host gut immune responses. We produced germ-free wild-type (GFWT) and conventionally reared wild-type (CRWT) animals (fig. S1) to examine further elucidation of the gut IMD-Relish pathway potential in the absence or presence of commensals. A large amount of the nuclear-translocated active form of Relish was detected in the intestine cells in the presence of commensals (CRWT flies) and was even more pronounced after gut infection with the Drosophila pathogen Erwinia carotovora carotovora-15 (Ec15) (Fig. 1A).

The well-characterized constitutive nuclear localization of Relish in the intestines of CRWT flies was almost completely abolished in the absence of commensals in GFWT or in antibiotics-treated CRWT flies. Similarily, it was not seen in flies carrying a mutation in the IMD-Relish pathway (CRDoid flies) (Fig. 1A). The expression levels of Relish-dependent immunosuppressive enzymes such as PGRP-SC and PGRP-LB (12, 13) were significantly higher in the guts of CRWT flies than in the guts of GFWT or CRDoid flies (Fig. 1B), which confirms that the IMD-Relish pathway was activated by commensals under normal gut conditions. However, the Relish-dependent immune effector molecules, such as AMP genes including Cecropin (Cec) and Diptericin (Dpt), were largely silenced in the CRWT gut despite chronic Relish activation. Similarly, no significant difference in AMP expression levels was observed between the GFWT and CRWT midguts (Fig. 1B).

Taken together, these results indicate that although commensal organisms of the gut can chronically induce a high level of local IMD-NF-κB pathway activation, only a subset of target genes (notably excluding AMP genes) are activated.

Role of Caudal in gut AMP repression. We next investigated the potential mechanisms of repression of the gut AMP genes to determine how the selective silencing toward commensals might occur. Currently, the cis-regulatory elements controlling epithelial AMP gene expression are poorly understood. It is known, however, that the NF-κB elements in the promoter regions responsible for nuclear factor kappa B (NF-κB)–Relish binding are essential for inducing epithelial AMP expression (14–16), whereas the CDREs, responsive elements for the homeobox transcription factor Caudal (Cad) responsible for Cad binding, are found to be critical for constitutive AMP expression in certain types of epithelia, such as salivary glands and the ejaculatory duct (18). The homeobox transcription factor Cad was originally identified on the basis of its regulatory role in the anteroposterior body axis formation of the Drosophila embryo (19, 20). Because Cad expression in postembryonic life is known to be mostly restricted to the intestine (19) (fig. S2), we analyzed the contribution of CDREs to gut AMP gene expression in vivo. To accomplish this, we used green fluorescent protein (GFP) reporter–expressing transgenic flies carrying a Cec promoter in which the CDREs were mutated (CecCDREmutGFP) and compared GFP expression with that of transgenic flies carrying the wild-type promoters fused to GFP (Cec-GFP) (Fig. 1C). We were unable to detect any Cec reporter activity in the midgut of Cec-GFP flies under normal conditions (Fig. 1C). Interestingly, however, Cec–CecCDREmutGFP flies were found to exhibit high constitutive expression of Cec reporter activity in the posterior midgut and the proventriculus in the absence of oral infection (Fig. 1C).

To test whether Cad was involved in the negative regulation of Cec expression through CDREs, we generated transgenic flies that carried the UAS-Cad-RNAi construct to mimic the loss of function (fig. S3) via RNA interference (RNAi). A spontaneous activation of all tested AMPs was observed in the gut of CRCad-RNAi flies under conventional conditions without microbial infection, which was not the case in the control flies (Fig. 1D). Furthermore, similar AMP derepression was also observed in Cad-RNAi flies with different GAL4 drivers (fig. S4). The role of Cad as a repressor was further confirmed by a strong expression of Dpt reporter activity observed in the gut of CRCad-RNAi flies carrying the Dpt-LacZ reporter (fig. S5). Cad-dependent AMP repression is highly tissue-specific because no AMP derepression was observed in the fat body of CRCad-RNAi flies (Fig. 1D). Introduction of Cad-RNAi had no effect on the expression of PGRP-SC and PGRP-LB (Fig. 1D), which indicates that any repressive role of Cad is restricted to a distinct

1Division of Molecular Life Science, Ewha Woman’s University and National Creative Research Initiative Center for Symbiosysystem, Seoul 120-750, South Korea. 2Laboratoire de BBMI, Institut Pasteur, Paris 75724, France. 3Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, South Korea. 4School of Life Science and Biotechnology, Kyungpook National University, Daegu 702-701, South Korea. 5Brain Korea 21 Program, Yonsei University College of Medicine, OPO Box 8044, Seoul 120-752, South Korea.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: lw@ewha.ac.kr
Fig. 1. Caudal acts as a gut-specific repressor for NF-κB–dependent AMP genes. (A) Nuclear-translocated active form of Relish (antibody to Relish, green) in posterior midgut (5- or 10-day-old flies), CRWT, conventionally reared wild-type flies; GFWT, germ-free wild-type flies; CRWT + Ab, antibiotics-treated CRWT flies as described in fig. S1; CRDredd, conventionally reared Dredd mutant flies; CRWT + Ecc15, natural gut infection with Ecc15. Nuclear staining was performed with 4’,6’-diamidino-2-phenylindole (DAPI). (B) Quantitative real-time PCR analysis of PGRP-SC, PGRP-LB, Diptericin (Dpt), and Cecropin (Cec) using dissected posterior midguts (without malpighian tubules) of 5-day-old flies. The target gene expression level in the tissues of GFWT flies was taken arbitrarily as 1. (C) CDREs are required for the repression of Cec expression (indicated by arrow) in the posterior midgut (upper panel) and the proventriculus (lower panel). A schematic diagram of the Cec promoter is also shown. (D) Quantitative real-time PCR analysis using posterior midguts or fat body. Genotypes of flies: CRCont (c729-GAL4/+) and CRCad-RNAi (c729-GAL4/+; UAS-Cad-RNAi/+). The target gene expression level in the gut or fat body of CRCont flies was taken arbitrarily as 1. Dpt, Diptericin; Cec, Cecropin; Drs, Drosomycin; Def, Defensin; Att, Attacin; Metch, Metchnikowin. (E) Quantitative real-time PCR analysis. Genotypes of flies (5-day-old GF or CR): Cont (c729-GAL4/+); Cad-RNAi (c729-GAL4/+; UAS-Cad-RNAi/+); Cad-RNAi + Dredd (DreddB118; c729-GAL4/+; UAS-Cad-RNAi/+); Cad-RNAi + TAK1 (TAK1; c729-GAL4/+; UAS-Cad-RNAi/+). The target gene expression level in the CRCont gut was taken arbitrarily as 1. (F) Overexpression of Cad can abolish infection-induced AMP expression. The target gene expression level in uninfected control gut was taken arbitrarily as 1. Natural gut infection for 6 hours with Ecc15 in (A), (D), and (F) was performed as described in (21); relative expression levels in (B), (D), (E), and (F) are expressed as means ± SD (P < 0.05) of three different experiments.

Fig. 2. Apoptosis assays reveal that AMP overexpression in the presence of commensal microbiota induces gut apoptosis. The posterior midguts of adult flies at different ages (A) and those of 10-day-old adult flies (B to D) were used. An apoptosis index was determined by dividing the number of apoptotic cells by the total number of cells and multiplying by 100. Values represent means ± SD (P < 0.05) of five independent experiments. The genotypes of Cad-RNAi flies with the c729-GAL4 driver in (A) and (C) were described in Fig. 1E. (A) Time course analyses of gut epithelial cell apoptosis in Cad-RNAi flies. (B) Apoptosis in Cad-RNAi flies with different GAL4 drivers: the intestine-specific Cad-GAL4 (Cad-GAL4/+; UAS-Cad-RNAi/+), and the strong ubiquitous Da-GAL4 (UAS-Cad-RNAi/Da-GAL4). Flies carrying only GAL4 driver were used as controls. (C) Apoptosis in CR and GF flies. The genotype of Cec + Dpt flies was c729-GAL4/UAS-Dpt; UAS-Cec/+ and the strong ubiquitous Cad-GAL4/UAS-Cad-RNAi/+ and the strong ubiquitous Da-GAL4/UAS-AmpGal4. Flies carrying only GAL4 driver were used as controls. (D) Apoptosis in CR flies overexpressing a single AMP. Fly genotypes: Cont (Da-GAL4/+); Cec (UAS-Cec/+; Da-GAL4/+); Dpt (UAS-Dpt/+; Da-GAL4/+). *ND, not detected.
subset of NF-κB–dependent genes such as AMPs. When we examined Cad-RNAi–induced AMP derepression in the IMD-Relish pathway mutant genetic backgrounds (CR\textsuperscript{Cad-RNAi} + Dredd or CR\textsuperscript{Cad-RNAi} + TAK1 flies) or in the absence of commensals (Gf\textsuperscript{Cad-RNAi} flies), the high level of AMP derepression was completely abolished (Fig. 1E).

These data show that Cad acts as a gut-specific transcriptional repressor exerting its antagonistic role in commensal-induced NF-κB–dependent AMP induction. Furthermore, the overexpression of Cad in the gut could abolish the infection-induced AMP expression (Fig. 1F).

Thus, it is likely that a dynamic equilibrium between Cad and Relish is one of the major mechanistic aspects determining the selective deployment of gut IMD-AMP pathway potential (21) (Fig. 1 and fig. S6). Because AMPs have a microbicidal effect against a broad spectrum of microorganisms, Cad-mediated AMP repression is likely required for establishing an optimal environment for the commensal microbiota.

Role of Cad in gut homeostasis. Several recent lines of evidence suggest that deregulation of the intestinal NF-κB pathway may be relevant to the etiology and pathology of many important diseases, including inflammatory bowel diseases (IBDs) (22–29). Given that IBDs typically involve apoptosis of intestinal cells, we examined whether cell death might be occurring in the gut of Cad-RNAi flies. Time-course analyses with adult flies showed that gut epithelial cell apoptosis was detected in Cad-RNAi flies at day 18, but not in control flies of the same age (Fig. 2A). Cell death was also observed in the intestines of CR\textsuperscript{Cad-RNAi} flies with different GAL4 drivers: c729-GAL4, Cad-GAL4, and Da-GAL4 (Fig. 2, A and B). Interestingly, gut epithelial cell apoptosis was abolished in the Gf\textsuperscript{Cad-RNAi} gut (Fig. 2C), which suggests the involvement of commensal organisms in Cad-RNAi–mediated gut pathology. To determine whether the high apoptosis level seen in the Cad-RNAi gut was due to secondary effects of AMP hyperactivation, we overexpressed two AMP genes (Cec and Dpt) to mimic the Cad-RNAi genotype. AMP overexpression in CR flies (CR\textsuperscript{Cec\textendash}Dpt) was sufficient to induce a high level of gut epithelial cell apoptosis (Fig. 2C). This was not the case in the absence of commensal microbiota (Gf\textsuperscript{Cec\textendash}Dpt) (Fig. 2C). When we overexpressed only a single AMP (Cec or Dpt) in CR flies (CR\textsuperscript{Cec} or CR\textsuperscript{Dpt}), similar gut epithelial cell apoptosis could be also observed (Fig. 2D). Overall, these in vivo results show that gut AMP overexpression in the presence but not in the absence of gut commensal microbes is sufficient to cause gut pathology.

Role of Cad in the gut commensal community structure. Because constitutive production of AMPs in the guts of Cad-RNAi flies likely affects the ecosystem of normal commensals, we next determined the dominant commensal species in the midgut of control (CR\textsuperscript{Cont}) and Cad-RNAi (CR\textsuperscript{Cad-RNAi}) flies. In wild-type flies, five commensal species dominate: Lactobacillus plantarum (LP), Lactobacillus brevis (LB), Acetobacter pomorum (AP), and two novel strains: Gluconobacter sp. strain EW707 (G707), and a bacterium in the family Acetobacteraceae, strain EW911 (A911) (21) (figs. S7 to S9 and table S1). All of these commensal bacteria, but not other bacteria such as Ecc15 and Escherichia coli, could persist in the gut, demonstrating their competences as commensal bacteria (Fig. 3A). Real-time polymerase chain reaction (PCR)–based quantitative analyses of each commensal (21) (figs. S9 to S11) clearly revealed that the commensal community structure of the Cad-RNAi flies differed from that of the control flies (Fig. 3B). Three bacteria—AP, LP, and LB—were commonly dominant in the gut of both control and Cad-RNAi flies (Fig. 3B). However, A911 [a dominant commensal member in controls, ~1.4 × 10\textsuperscript{5} colony-forming units (CFUs) per gut] was markedly diminished and maintained at a very low level in the Cad-RNAi gut (~900 CFUs per gut), whereas G707 (a minor commensal member in the control gut, ~800 CFUs per gut) emerged as a dominant commensal in the Cad-RNAi gut (~1.7 × 10\textsuperscript{4} CFUs per gut) (Fig. 3B). Time-course analyses with Cad-RNAi flies showed that loss of A911 was visible from as
GFCont or GFCad-RNAi embryos and maintained each of the isolated commensal bacteria into involved in the gut pathology.

when GF flies were subjected to gut infection with (Fig. 4A), and G707 did not induce the apoptosis in the fat body (Fig. 4A), but not when the single organism was G707 (Fig. 4A), and G707 was pathogenic to the host when GF flies were subjected to gut infection with G707 (Fig. 4B). These results show that G707 is indeed pathogenic in a gut-specific manner when allowed to become the dominant gut microbe.

To further confirm that reorganization of the gut microbiota composition seen in the Cad-RNAi flies was due to the constitutive overexpression of microbial AMPs, we tested whether the artificial shift of gut microbiota composition could occur as a result of overexpression of AMPs. We found that overexpression of Cec and Dpt (CR_Cec+Dpt) was sufficient to induce the modification of commensal community (i.e., G707 dominance and a loss of A911), as in the case of the Cad-RNAi guts (Fig. 4C). Similar results could also be observed when we overexpressed only a single AMP (CR_Cec or CR_Dpt) (Fig. 4C). To confirm that the loss of A911 was due to the high sensitivity of this strain to AMP, we performed an in vitro antibacterial test with synthetic Cec A1 and determined the minimum inhibitory concentration (21). The result showed that A911 was highly susceptible to low concentrations of synthetic Cec A1, whereas all other commensals as well as G707 exhibited relatively high resistance to Cec A1 (Table 1). Thus, we conclude that gut AMP overexpression in Cad-RNAi flies acts as a distinct selection pressure on different commensal microbes, resulting in modification of the commensal community structure and pathogenic conditions in the gut.

Role of the normal commensal community in gut homeostasis. Because G707 is normally present at a very low level in the wild-type commensal community, we investigated whether the normal wild-type commensal community structure could antagonize the dominance of G707. To accomplish this, we introduced G707 by oral feeding into the gut of either GF<sub>WT</sub> or CR<sub>WT</sub> flies and then examined its persistence. The results indicated that G707 bacteria could persist in the absence of other commensal organisms in GF<sub>WT</sub> flies but disappeared rapidly and were maintained at a low level in the presence of the normal commensal community in CR<sub>WT</sub> flies (Fig. 5A).

Consistent with these results, we observed a high-level gut epithelial cell apoptosis in the G707-challenged GF<sub>WT</sub> flies, but not in the G707-challenged CR<sub>WT</sub> flies (Fig. 5A), which demonstrates the role of normal commensal community structure in gut homeostasis by maintaining G707 at a low level.

Given the numerical inferiority of A911 in G707-dominant gut environments, we investigated whether the presence of A911 was suf-
sufficient to suppress G707 dominance in the gut. To accomplish this, we generated monoassociated flies by introducing isolated A911 into GF WT embryos and maintaining this presence until the adult stage. When G707 was introduced into these monoassociated flies through oral feeding, the G707 bacteria rapidly decreased to a low level in the presence of A911, whereas a high initial G707 level was able to persist in the absence of A911 (in the case of GF WT flies) (Fig. 5A). After G707 introduction, a high apoptosis index was observed in the gut of the GF WT flies, but not in the gut of A911 monoassociated flies (Fig. 5A). This result shows that the presence of A911 is sufficient to prevent G707 dominance, and that the loss of A911 in the AMP-overexpressing genotype flies (such as Cad-RNAi flies or Cec- or Dpt-overexpressing flies) is likely to be responsible for G707 dominance.

Taken together, these results reveal that Cad acts as a critical host factor that maintains the immune homeostasis responsible for preservation of the normal commensal community structure. Failure of the balanced regulation of AMP, as in the case of Cad-RNAi flies, can act as a novel selection pressure leading to modification of the gut microbiota. Because G707 is a highly pathogenic organism (Fig. 4, A and B), the G707-dominating host genotype acts as an initial cause of gut apoptosis, whereas the dominance of G707 acts as a direct cause of gut apoptosis.

Role of Cad in host physiology. The apoptosis of gut cells seen in Cad-RNAi flies with different GAL4 drivers was accompanied by elevated mortality (Fig. 5E). The high mortality of CR Cad-RNAi flies was significantly ameliorated in the absence of commensal (GF Cad-RNAi) flies (Fig. 5E) or in the presence of commensals under the IMD pathway mutant genetic backgrounds (CR Cad-RNAi + Dredd) (Fig. S14). The normal survival rate could be also observed in GF Cad-RNAi flies stably associated with major commensal microbiota (AP, LP, LB, and A911) excluding G707, which implies the involvement of G707 in Cad-RNAi-mediated host mortality (Fig. 5E). Furthermore, the c-Jun N-terminal kinase pathway and interleukin-1β converting enzyme were shown to be involved in the microbiota-induced gut apoptosis and mortality process of the Cad-RNAi flies (Fig. S15).

To demonstrate that the initial cause of death in the Cad-RNAi flies was the reduced expression of Cad, we attempted an in vivo rescue experiment. Restoration of the basal AMP level (Fig. 5B), healthy microbiota community structure (Fig. 5C), reduced apoptosis (Fig. 5D), and normal host survival levels (Fig. 5E) could be achieved in the Cad-RNAi flies by genetic reintroduction of Cad (CR Cad-RNAi + Cad). Taken together, our results show that the intestinal homeobox Cad gene is responsible for the delicate immune homeostasis in the microbiobacteria-gut tissue, which is essential for preservation of the normal microbiota community, gut homeostasis, and host survival.

**Fig. 5.** Cad is indispensable for immune homeostasis in preserving the indigenous commensal community and host health. (A) Normal commensal community structure is important to suppress G707 dominance in the gut. Germ-free wild-type flies (GF WT), wild-type flies carrying normal commensal microbiota (CR WT), and wild-type flies monoassociated with A911 (A911-GF WT) were used. G707 was introduced to these flies (3 days old) through oral feeding for 48 hours. Left panel: Real-time PCR-based analysis to quantify G707 in the gut was performed at 1, 5, and 10 days after G707 feeding. Right panel: Apoptosis assay was performed as described in Fig. 2. Values represent means ± SD (P < 0.05) of five independent experiments. (B to E) Restoration of basal AMP levels (B), normal commensal community structure (C), reduced apoptosis (D), and normal survival levels (E) could be achieved in the Cad-RNAi flies by genetic reintroduction of Cad. The posterior midguts of 18-day-old adult flies were used for analyses of commensal community structure. Apoptosis assay was performed as described in Fig. 2. Genotypes of CR flies: Cont (c729-GAL4/+-); Cad-RNAi (c729-GAL4/+; UAS-Cad-RNAi/+-); Cad-RNAi + Cad (c729-GAL4/UAS-Cad; UAS-Cad-RNAi/+). GF Cond and GF Cad-RNAi flies were also used. AP/A911/LP/LB-GF Cad-RNAi flies carrying four major commensals AP, A911, LP, and LB, excluding G707 were generated by colonizing GF embryos with commensals as described in (21). Values represent means ± SD (P < 0.05) of three independent experiments. *ND, not detected.
Quantum Phase Extraction in Isospectral Electronic Nanostructures

Christopher R. Moon, Laila S. Mattos, Brian K. Foster, Gabriel Zeltzer, Wonhee Ko, Hari C. Manoharan

Quantum phase is not directly observable and is usually determined by interferometric methods. We present a method to map complete electron wave functions, including internal quantum phase information, from measured single-state probability densities. We harness the mathematical discovery of drum-like manifolds bearing different shapes but identical resonances, and construct quantum isospectral nanostructures with matching electronic structure but divergent physical structure. Quantum measurement (scanning tunneling microscopy) of these “quantum drums”—degenerate two-dimensional electron states on the copper(111) surface confined by individually positioned carbon monoxide molecules—reveals that isospectrality provides an extra topological degree of freedom enabling robust quantum state transplantation and phase extraction.

The local structure of wave functions can now be experimentally determined in many materials. However, just as bonding in molecules or conductivity in solids depends not only on the magnitude of the orbital wave functions but also on the phase, the electronic properties and dynamics of nanostructures critically depend on determining both the magnitude and the phase of wave functions. In a classical system, measuring the phase of a standing wave is trivial, but the internal phase of a quantum wave function $\psi$ is not observable; only the probability density $|\psi|^2$ can be determined from direct measurement. To determine phase, some form of additional information is required.

Recently, quantum phase has been inferred in several experiments, including spectral interferometry of Rydberg wave packets (1) and tomography of molecular orbitals (2). Such experiments are performed on atoms or molecules in the gas phase and require ultrafast measurements of the response of a quantum state to an impinging wave or excitation. In solid-state systems, phase-sensitive measurements have been performed in quantum dots and rings (3) and with computational post-processing of diffraction patterns (4). All of these experiments retrieve the underlying phase differences through interferometry once a reference beam and geometry are defined.

Here, we present a noninterferometric method to map the internal quantum phase of a solitary, time-independent state by harnessing the topological property of isospectrality as the additional degree of freedom. We create a particular pair of geometric shapes with matching spectral properties. We then show that the complete phase information of wave functions in both structures can be experimentally determined. 

1Department of Physics, Stanford University, Stanford, CA 94305, USA. 2Department of Electrical Engineering, Stanford University, Stanford, CA 94305, USA. 3Department of Applied Physics, Stanford University, Stanford, CA 94305, USA.

*To whom correspondence should be addressed. E-mail: manoharan@stanford.edu