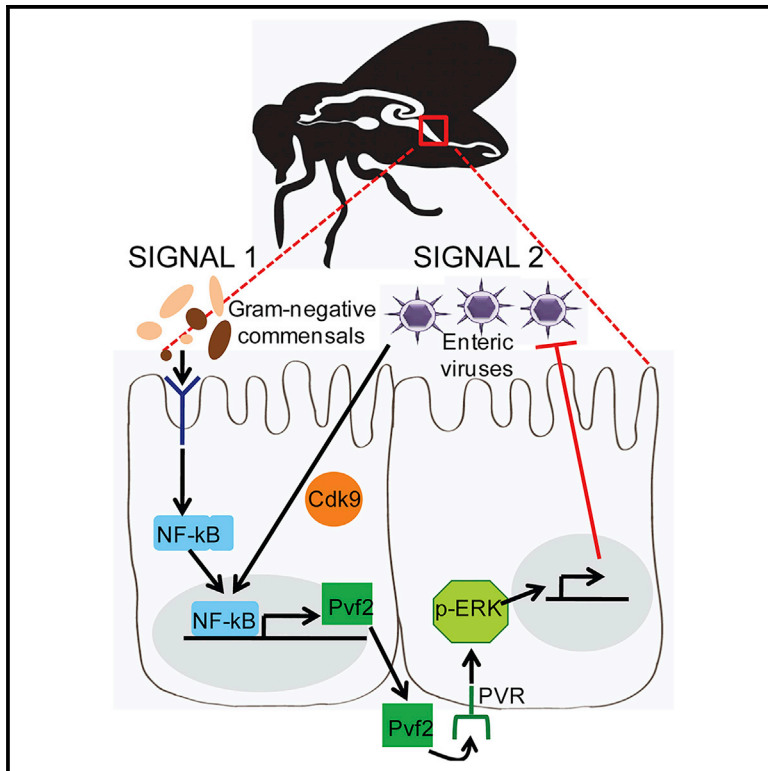


# Cell Host & Microbe

## Microbiota-Dependent Priming of Antiviral Intestinal Immunity in *Drosophila*

### Graphical Abstract



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### In Brief

Many pathogens, including viruses, are orally acquired and must overcome intestinal immunity to infect the gastrointestinal tract. Sansone et al. demonstrate that a two-signal system, consisting of microbiota-dependent priming and virus-dependent sensing, is required to induce antiviral ERK signaling in enterocytes that blocks enteric viral infections.

### Highlights

- The microbiota primes antiviral signaling in the intestinal epithelium
- Gram-negative commensal-dependent NF-κB signals are required for antiviral responses
- NF-κB cooperates with a virus-induced Cdk9-dependent signal to induce Pvf2
- Pvf2, a secreted ligand, binds the PVR receptor to induce antiviral ERK signaling



# Microbiota-Dependent Priming of Antiviral Intestinal Immunity in *Drosophila*

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## SUMMARY

Enteric pathogens must overcome intestinal defenses to establish infection. In *Drosophila*, the ERK signaling pathway inhibits enteric virus infection. The intestinal microflora also impacts immunity but its role in enteric viral infection is unknown. Here we show that two signals are required to activate antiviral ERK signaling in the intestinal epithelium. One signal depends on recognition of peptidoglycan from the microbiota, particularly from the commensal *Acetobacter pomorum*, which primes the NF- $\kappa$ B-dependent induction of a secreted factor, Pvf2. However, the microbiota is not sufficient to induce this pathway; a second virus-initiated signaling event involving release of transcriptional paused genes mediated by the kinase Cdk9 is also required for Pvf2 production. Pvf2 stimulates antiviral immunity by binding to the receptor tyrosine kinase PVR, which is necessary and sufficient for intestinal ERK responses. These findings demonstrate that sensing of specific commensals primes inflammatory signaling required for epithelial responses that restrict enteric viral infections.

## INTRODUCTION

Enteric viral pathogens are widespread. Humans are commonly infected with enteroviruses, and these infections are associated with a wide variety of clinical manifestations ranging from asymptomatic to meningitis (Abzug, 2014; Jubelt and Lipton, 2014; Muehlenbachs et al., 2015). Recent epidemiological evidence indicates that enteric viruses are the leading cause of foodborne disease in the USA and worldwide are a major group of waterborne disease agents (Atreya, 2004; Koo et al., 2010; Sair et al., 2002). Enteroviruses are a widespread class of picornaviruses that infect organisms from insects to humans. The picorna-like virus of *Drosophila*, *Drosophila* C virus (DCV), is a widespread pathogenic enterovirus of fruit flies (Jousset, 1976). Arthropod-borne viruses (arboviruses) are another group of viruses of global importance. Infection of the insect vector occurs orally during the blood meal, while infection of vertebrate hosts is through an insect bite (Attardo et al., 2005; Hansen

et al., 2014; Raikhel and Dhadialla, 1992). Viruses within this blood meal infect intestinal epithelial cells to establish infection, as is the case for many enteric infections in mammals (Davis and Engström, 2012; Steinert and Levashina, 2011; Weaver and Barrett, 2004). Moreover, there has been a resurgence of vector-borne viral pathogens, which have become an increasing source of worldwide morbidity and mortality in humans and livestock. In particular, dengue virus (DENV), a member of the *Flaviviridae* family, is a re-emerging arbovirus that infects >300 million people and causes ~250,000 deaths annually (Bhatt et al., 2013).

It has long been recognized that the gut represents a formidable immune barrier against enteric viral infections in both vertebrates and insects. The high barrier presented by the gastrointestinal tract causes most studies on human enteric viruses in mice to rely on intraperitoneal injection (Bopegamage et al., 2005; Gill et al., 2011; Mossel and Ramig, 2002; Nagler-Anderston, 2001). Arboviruses within the blood meal must also overcome barrier immunity to establish infection in the insect (Weaver and Barrett, 2004). Experimentally, this infection barrier is well described: oral infection of mosquitoes that are not the natural vector is usually non-productive; however, bypassing the gut by injecting the virus in the body cavity allows the virus to establish infection that can even be transmitted to vertebrates (Kingsolver et al., 2013; Tabachnick, 2013; Xu and Cherry, 2014). While the intestinal environment is clearly restrictive to viral infection from insects to humans, few molecular mechanisms are known.

The gut is a complex environment, housing an extensive microbiota that influences homeostasis and nutrient uptake. Recently, there has been an increasing appreciation that the commensals that inhabit the intestine are essential players in immunity across hosts (Buchon et al., 2013a; Charroux and Royet, 2012; Lee and Brey, 2013; Sommer and Bäckhed, 2013). Indeed, the microbiota and innate immune system are constantly engaged and impact infection in the gut (Cirimotich et al., 2011; Pang and Iwasaki, 2012; Ramirez et al., 2012; Schaffer et al., 1963; Xi et al., 2008). However, the molecular links between the microbiota and immunity are only beginning to be defined. Understanding the role of the microbiota in the context of viral infection may reveal strategies to restrict enteric infections.

To explore the mechanisms involved in oral acquisition of viral pathogens, we developed an oral model of viral infection using the genetically tractable organism *Drosophila melanogaster* (Xu et al., 2013). We found, as has been shown in vectors and murine systems, that the intestine is highly restrictive; however, loss of ERK signaling in the intestinal epithelium, specifically

in enterocytes, significantly increases susceptibility to the *Drosophila* enteric picorna-like virus DCV (Xu et al., 2013). We also tested human arboviruses from three different families that are orally acquired in insects and observed that ERK is restrictive. Importantly, we found that the intestinal epithelium rapidly responds to viral infection by inducing the ERK pathway (Xu et al., 2013). Since these viruses are diverse, but regulated similarly, our data suggest that the ERK pathway is broadly antiviral against orally acquired viruses.

In this study, we set out to determine how the ERK pathway was regulated in the gut to control viral infection. We found that the ligand Pvf2 is induced upon viral infection and activates the receptor tyrosine kinase (RTK), PVR, which is required for activation of the antiviral ERK pathway in enterocytes. Moreover, we found that Pvf2 induction is regulated by the microbiota; gram-negative commensals are sensed by enterocytes priming NF- $\kappa$ B-dependent Pvf2 expression. In the absence of the microbiota, the animals are more susceptible to oral challenge and this can be overcome by ectopically expressing Pvf2 or by mono-association with *Acetobacter pomorum*, a gram-negative commensal that activates Pvf2, but not *Lactobacillus brevis*, a gram-positive commensal that does not induce Pvf2. A second signal is required that is dependent on sensing virus. We had previously defined a pausing-dependent transcriptional program in flies (Xu et al., 2012) and we now show that this pathway is also required for virus-dependent Pvf2 induction. Taken together, these results clearly demonstrate that sensing of specific components of the microbiota coupled with viral signals are integrated to play an essential role in the control of enteric viral infection of a broad range of viruses.

## RESULTS

### PVR Is Required for Antiviral Defense

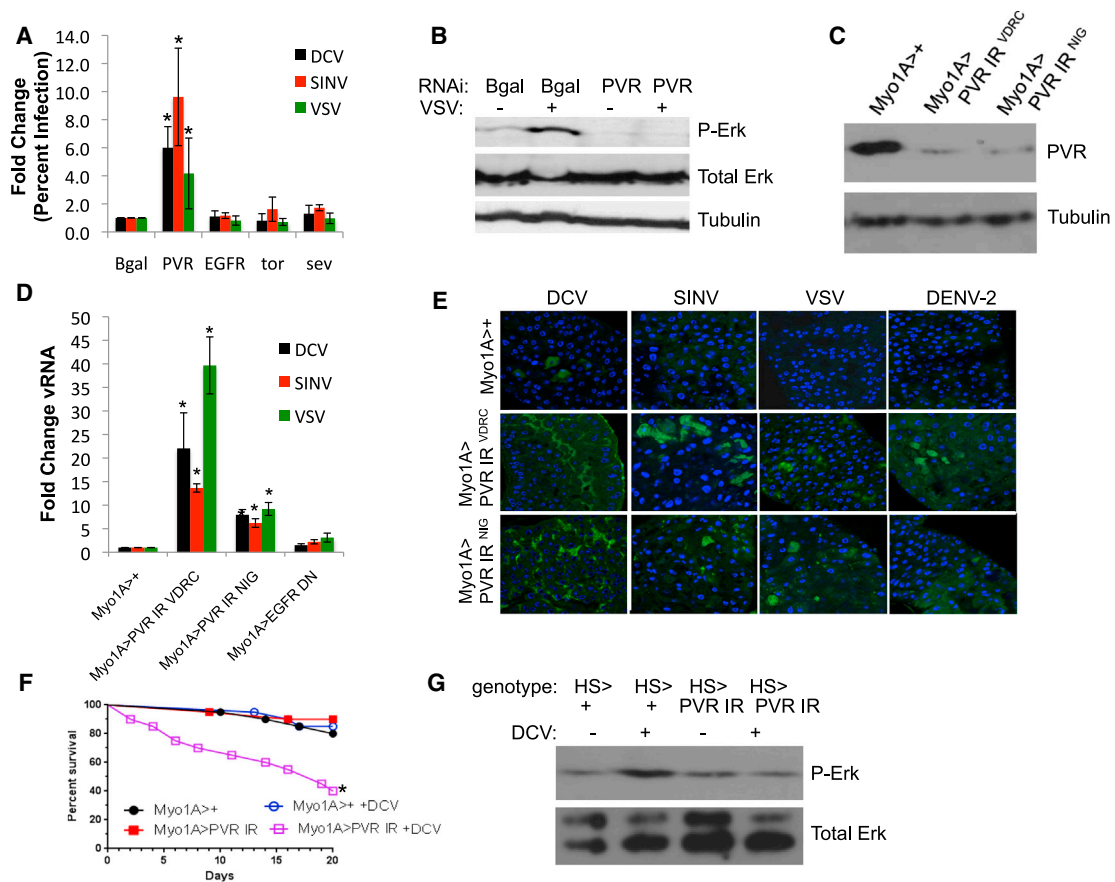
The canonical ERK signaling pathway is initiated by secreted factors binding to RTKs, which activates a three-tiered phosphorylation cascade, culminating with phosphorylation of ERK (Sundaram, 2013). The *Drosophila* genome encodes 21 RTKs (Sopko and Perrimon, 2013), and to determine the receptor responsible for activating the antiviral ERK pathway in the *Drosophila* intestine, we screened a panel of RTKs in vitro using RNAi for their role in antiviral defense against a panel of viruses that we previously found to be restricted by the ERK pathway. This included Sindbis virus (SINV), Vesicular stomatitis virus (VSV), and DCV. VSV and SINV are arboviruses belonging to two disparate families (*Alphaviridae* and *Rhabdoviridae*, respectively). Their natural cycle involves transmission between insect vectors and vertebrate hosts, but they do not naturally infect *Drosophila*. DCV is a natural *Drosophila* enteric pathogen similar to picornaviruses (Jousset, 1976). We found that only when PVR (platelet derived growth factor and vascular endothelial growth factor receptor) was depleted, we observed a significant increase in infection with all three viruses in cell culture (Figure 1A). We previously showed that virus infection is sensed in *Drosophila* leading to the activation of ERK signaling (Xu et al., 2013), and using an antibody that recognizes activated *Drosophila* ERK (phospho-ERK), we observed that the virus-induced increase in phospho-ERK was dependent on PVR in vitro (Figure 1B; Figure S1A).

We next examined the requirement of PVR in the gut. The *Drosophila* intestine, similar to the mammalian intestine, is a tubular epithelium composed of a monolayer of cells with the absorptive enterocytes lining >95% of the surface area. We previously found that ERK is specifically required in enterocytes (Xu et al., 2013), which is the known target of many enteric viruses, including picornaviruses in humans and arboviruses in their insect vectors (Blair, 2011; Davis and Engström, 2012; Franz et al., 2015; Mordstein et al., 2010; Peterson and Artis, 2014). Using in vivo RNAi specifically expressing in the intestinal epithelial cells (Myo1A-GAL4 driver), we depleted PVR using two independent RNAi lines and verified efficient knockdown of PVR (Figure 1C; Figure S1B). We found that these flies had a normal lifespan (Figure 1F) with no barrier dysfunction (Figure S1C) (Rera et al., 2011). Upon challenge with DCV, SINV, VSV, or DENV flies with PVR-depleted intestinal epithelial cells had increased viral infection in the intestine as measured by RT-qPCR (Figure 1D; Figure S1D) and confocal microscopy (Figure 1E) with the infection largely in the posterior midgut (Figures S1E and S1F). As a control, we expressed a dominant-negative Epidermal Growth Factor Receptor (EGFR DN), another RTK endogenously expressed in the intestine (Buchon et al., 2010), which had no impact on viral infection (Figure 1D). To further confirm these results, and bypass any developmental requirements, we used a heat shock-inducible driver to deplete PVR only in adult animals prior to challenge. Upon heat shock, we found again that knockdown of PVR resulted in a significant increase in DCV infection in the intestine (Figure S1G). Moreover, we found that loss of PVR in the intestinal epithelium had a major impact on immunity. First, PVR-depleted animals challenged with DCV now succumbed to infection, converting a largely non-pathogenic infection into a lethal one (Figure 1F). Second, we found that PVR was required for virus-induced ERK signaling in the gut (Figure 1G; Figure S1H). Altogether, our data show that PVR is a receptor required for antiviral ERK signaling in the intestinal epithelium.

### Pvf2 Is Required for Antiviral Defense

RTKs are activated by secreted ligands. PVR has three known ligands: Pvf1, Pvf2, and Pvf3 (Cho et al., 2002; Duchek et al., 2001) and RNAi in cell culture revealed that Pvf2 was required for antiviral defense against DCV, SINV, and VSV (Figure 2A). We orally challenged flies mutant for Pvf2 (Pvf2<sup>c06947</sup>) and found that they are more susceptible to DCV, SINV, VSV, and DENV infection as measured by RT-qPCR (Figure 2B) and confocal microscopy (Figure 2C). In contrast, flies mutant for Pvf1 (Pvf1<sup>EP1624</sup>) do not display a change in viral infection in the intestine (Figure S2A). Moreover, Pvf2 mutants have increased lethality upon oral infection with DCV (Figure 2D). Furthermore, we found that Pvf2 is required for DCV-induced phospho-ERK signaling in the intestine (Figure 2E; Figure S2B).

Next, we tested whether Pvf2 induction was sufficient to induce antiviral ERK signaling in the gut. Here, we ectopically expressed Pvf2 in either the intestinal epithelium (Myo1A) or with a heat shock-inducible driver (hs) and confirmed that expression of Pvf2 resulted in an increase in basal phospho-ERK levels (Figures S2C–S2F). Next, we challenged these flies with DCV and observed decreased infection (Figure 2F; Figure S2G). We were unable to test the other viruses because we cannot detect



**Figure 1. PVR Is Required for Antiviral Defense in the *Drosophila* Intestine**

(A) *Drosophila* cells treated with the indicated dsRNAs were challenged with the indicated viruses and monitored by automated microscopy and image analysis. Percent of infected cells was quantified and normalized to control for three independent experiments with mean  $\pm$  SD shown; \* $p$  < 0.05.  
 (B) Representative immunoblot of *Drosophila* cells treated with the indicated dsRNAs and infected with VSV for 30 min.  
 (C) Representative immunoblot analysis of 20 pooled intestines from control (Myo1A > +) or PVR-depleted guts.  
 (D) Flies of the indicated genotypes were infected with the indicated viruses. RT-qPCR analysis of viral RNA normalized to rp49 and shown relative to control (Myo1A > +) from 15 pooled intestines 7 dpi with mean  $\pm$  SD;  $n \geq 3$ ; \* $p$  < 0.05.  
 (E) Representative confocal images of midguts from Myo1A > + or two independent RNAi lines to deplete PVR (Myo1A > PVR IR<sup>VDRC</sup> or NIG) infected with the indicated viruses analyzed 7 dpi (DCV, SINV, and VSV) or 10 dpi (DENV-2) (40 $\times$ ; virus, green; nuclei, blue).  
 (F) Percent survival of control (PBS-fed) or infected (DCV-fed) Myo1A > + or Myo1A > PVR IR<sup>NIG</sup> flies ( $n = 3$ , \* $p = 0.0026$ , log-rank test).  
 (G) Representative immunoblot analysis of 20 pooled intestines from control (HS > +) or PVR-depleted guts (HS > PVR IR<sup>NIG</sup>) at 2 hpi. See also Figure S1.

infection in wild-type flies. We next verified that Pvf2 is upstream of ERK, by challenging flies ectopically expressing Pvf2 in the presence and absence of the ERK inhibitor U0126 (Xu et al., 2013). Treatment with U0126 led to increased infection, which could not be suppressed by ectopic Pvf2 expression (Figure 2F). Therefore, Pvf2 is necessary and sufficient to induce the antiviral ERK pathway in the intestine.

### Pvf2 Is Induced by Viral Infection

Since we observed induction of ERK signaling upon infection that was dependent upon Pvf2, we hypothesized that Pvf2 is regulated during viral infection. We first monitored Pvf2 levels using transgenic flies that carry a lacZ reporter downstream of the endogenous Pvf2 promoter (Choi et al., 2008). Upon oral infection, we observed induction of lacZ in the posterior midgut (Figure 3A). This is the region of the gut where we observe the highest level of viral infection (Figures S1E and S1F) and that is

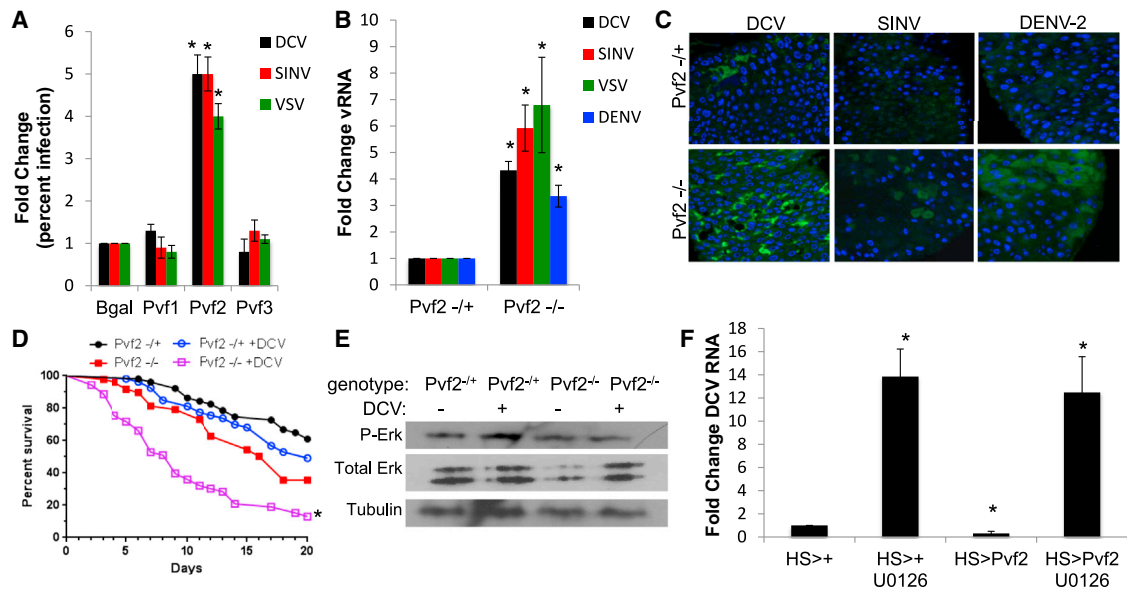
also the most responsive to the microbiota and bacterial infections (Bosco-Drayon et al., 2012; Broderick et al., 2014; Buchon et al., 2013a; Neyen et al., 2012).

Next, we monitored Pvf2 by RT-qPCR in the intestine following DCV infection over a wide time course. We observed a significant increase in Pvf2 mRNA 1 hpi that was highest at 4 hpi and returned to baseline by 24 hpi (Figure 3B). Moreover, we observed a significant increase in Pvf2 when flies were orally challenged with VSV (Figure 3C). This transcriptional induction was specific to Pvf2, since we did not observe a significant increase in Pvf1 or Pvf3 following oral challenge (Figures 3D and 3E).

### Pvf2 Expression Is Regulated by the Microbiota

We set out to determine how Pvf2 is regulated in response to infection. It was previously shown that treatment with *E. coli* for 1 hr can induce Pvf2 in cultured insect cells and that this was through the inflammatory Imd signaling pathway (Bond





**Figure 2. Pvf2 Is Required For Antiviral Defense in the *Drosophila* Intestine**

(A) *Drosophila* cells treated with the indicated dsRNAs were challenged with the indicated viruses and monitored by automated microscopy and image analysis. Percent of infected cells was quantified and normalized to control with mean  $\pm$  SD;  $n = 3$ ;  $*p < 0.05$ .  
 (B) Control (Pvf2<sup>-/+</sup>) or Pvf2 mutant (Pvf2<sup>-/-</sup>) flies were challenged with the indicated viruses. RT-qPCR analysis of viral RNA normalized to rp49 and shown relative to Pvf2 sibling controls from 15 pooled intestines 7 dpi (DCV, SINV, and VSV) or 10 dpi (DENV-2) with mean  $\pm$  SD;  $n \geq 3$ ;  $*p < 0.05$ .  
 (C) Representative images of midguts from sibling control or Pvf2 mutant flies infected with the indicated viruses analyzed 7 dpi (DCV and SINV) or 10 dpi (DENV-2) (40 $\times$ ; virus, green; nuclei, blue).  
 (D) Percent survival of control (PBS-fed) or infected (DCV-fed) Pvf2 mutants or heterozygous sibling controls. ( $n = 3$ ;  $*p < 0.01$ , log-rank test).  
 (E) Representative immunoblot analysis of 20 pooled Pvf2 mutant or heterozygous sibling control intestines at 2 hpi.  
 (F) RT-qPCR analysis of viral RNA normalized to rp49 and shown relative to control (HS>+) from 15 pooled intestines 7 dpi. Mean  $\pm$  SD;  $n = 4$ ;  $*p < 0.05$ . See also Figure S2.

and Foley, 2009). This suggested that bacterial peptidoglycan was the stimulant and, therefore, we reasoned that the endogenous microflora might regulate intestinal Pvf2. First, we tested whether bacterial products from *Drosophila* commensals could induce Pvf2 in vitro. The major commensals in the *Drosophila* intestine are: *Acetobacter pomorum*, *Acetobacter tropicalis*, *Lactobacillus brevis*, and *Lactobacillus plantarum* (Broderick and Lemaitre, 2012; Wong et al., 2011). We found that supernatants from *E. coli* and the gram-negative commensals *A. pomorum* and *A. tropicalis* strongly induced Pvf2 in cell culture, while the gram-positives *L. plantarum* and *L. brevis* did not (Figure 4A). Moreover, the relative levels of Pvf2 induction by these commensals correlates with activation of the Imd pathway as measured by the production of the antimicrobial peptide mRNA diptericin (Figure S3A).

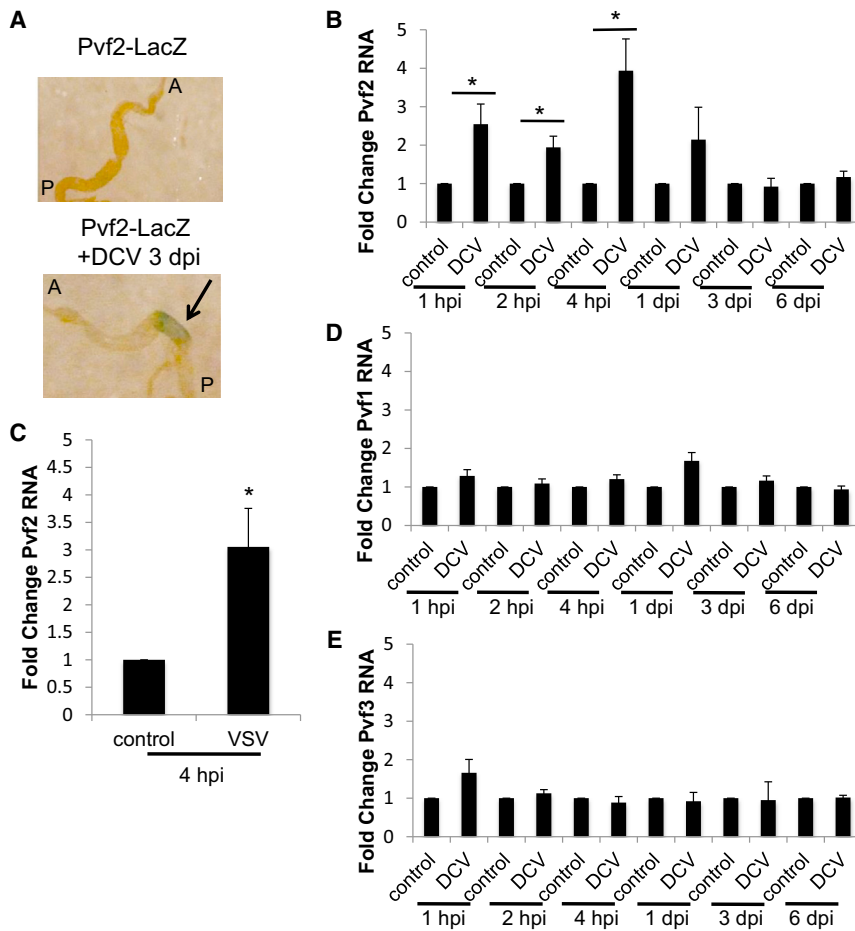
These data suggested that the microbiota, and in particular gram-negatives, might be playing a role in Pvf2 regulation during viral infection. We set out to test this hypothesis by manipulating the endogenous microbiota. First, we used a cocktail of antibiotics to ablate the microbiota and observed a significantly decreased bacterial load (>2.9 log decrease in CFU/gut; Figure S3B) and observed no defect in barrier function (Figure S3C). Second, we raised germ-free flies, verified that these flies had no detectable bacteria (Figure S3D), and observed normal barrier function (Figure S3E). We measured the basal levels of Pvf2 in the microbiota-depleted intestine and observed decreased Pvf2 mRNA levels as measured by RT-qPCR (Figures 4B and

4C). We also observed reduced basal phospho-ERK levels in antibiotic-treated intestines (Figure 4D; Figure S3F).

### The Microbiota Is Required for Intestinal Antiviral Defense and *A. Pomorum* Is Sufficient to Confer Intestinal Antiviral Immunity

If the microbiota regulates Pvf2, then loss of the microbiota would lead to increased enteric viral infection. First, we orally challenged antibiotic-treated adult flies with DCV or VSV and measured viral replication and found that antibiotic-treated flies were more susceptible to viral infection (Figures 4E and 4F). Second, we orally challenged germ-free flies (gf) and found that these flies displayed a significant increase in viral infection compared to conventionally reared animals (cv) (Figure 4G).

Therefore, the microbiota is required for antiviral defense and specific components of the microbiota efficiently activate Pvf2 expression in cell culture. These data suggest that the specific components of the microbiota that activate Pvf2 mediate this response. We thus hypothesized that *A. pomorum*, which potently induces Pvf2, would restore antiviral immunity to flies lacking a microbiota, while *L. brevis*, which is a poor inducer of Pvf2, would not (Figure 4A). To test this, we used a cocktail of antibiotics to ablate the microbiota and then monoassociated with *A. pomorum* or *L. brevis*. First, we determined the impact of monocolonization on Pvf2 induction in vivo. Consistent with our cell culture experiment, flies monoassociated with *A. pomorum* but not *L. brevis* had detectable Pvf2 expression



**Figure 3. Pvf2 Expression Is Induced by Viral Infection**

(A) Flies carrying a Pvf2 promoter-driven lacZ reporter (Pvf2-lacZ) were challenged with DCV and stained for beta-galactosidase activity. A representative image of the posterior midgut and arrow indicates DCV induced lacZ expression (A, anterior; P, posterior).

(B–E) RT-qPCR analysis of Pvf2 (B and C), Pvf1 (D), or Pvf3 (E) mRNA normalized to rp49 and shown relative to control from 15 pooled intestines infected with DCV (B, D, and E) or VSV (C) and isolated at the indicated time post infection. Mean  $\pm$  SD;  $n \geq 3$ ; \* $p < 0.05$ .

also challenged three different mutants in the Imd pathway: imd (*imd*<sup>1</sup> [the *Drosophila* homolog of FADD]), Tak1 (*Tak1*<sup>2</sup> [the *Drosophila* homolog of TAK1]), and Rel (*Rel*<sup>E38</sup> [the NF- $\kappa$ B transcription factor]). We found that all three mutants display a significant increase in viral infection in the intestine (Figures 6B–6C; Figures S4A and S4B). Since the Imd component Tak1 induces the JNK pathway (Silverman et al., 2003; Takatsu et al., 2000), and others have found that JNK can regulate Pvf2 (Bond and Foley, 2009), we also challenged flies expressing a dominant-negative form of JNK (*bsk*<sup>DN</sup>). We expressed *bsk*<sup>DN</sup> either in the intestinal epithelium or ubiquitously upon heat shock and found that inhibition of JNK signaling did not impact viral infection in

the intestine (Figure 5A). Furthermore, we challenged the monoassociated flies with DCV. We found that *A. pomorum* but not *L. brevis* could completely restore antiviral immune function (Figure 5B).

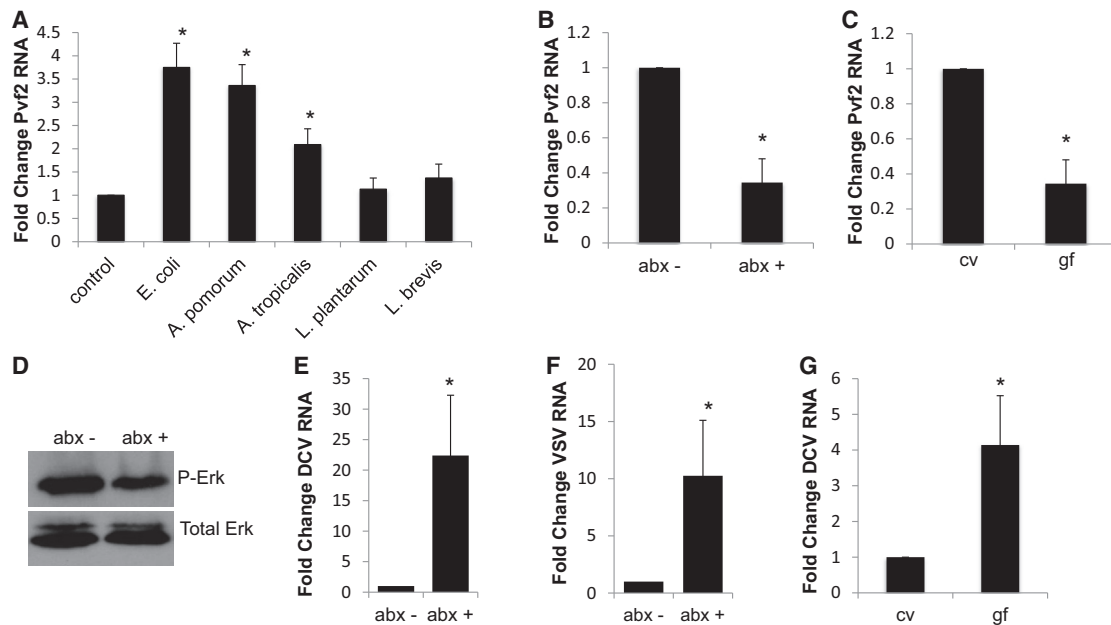
Since we used supernatants from the bacteria to induce Pvf2 in cell culture and a previous study found that peptidoglycan of *E. coli* was sufficient to induce Pvf2 in cell culture (Bond and Foley, 2009), we tested whether heat-killed bacteria could mediate the antiviral response. Indeed, we observed that flies monoassociated with heat-killed *A. pomorum* but not *L. brevis* had detectable Pvf2 expression in the intestine (Figure 5A). Furthermore, we found that heat-killed *A. pomorum* but not *L. brevis* could completely restore antiviral immune function (Figure 5B).

### The Imd Pathway Is Required for Antiviral Defense and Virus-Induced Pvf2 Expression

Since we found that heat-killed bacteria could protect flies, our data suggested that a bacterial PAMP was mediating the antiviral activity. Insects encode the Toll and Imd pathways that sense microbes and converge on NF- $\kappa$ B activation, but studies suggest that the Imd but not the Toll pathway is active in the intestine (Lee and Brey, 2013). Thus, we tested whether the Toll or Imd pathway plays a role in enteric viral infection. We challenged the Toll pathway mutant *dMyD88* (*dMyD88*<sup>EP(2)2133</sup>), which did not display altered susceptibility to infection (Figure 6A). We

the intestine (Figures S4C and S4D), suggesting a specific role for Imd-dependent NF- $\kappa$ B activation in antiviral defense. These data are consistent with previous studies that suggested that the posterior midgut, where we observed Pvf2 expression, is the region of the gut most responsive to the Imd pathway (Bosco-Drayon et al., 2012; Broderick et al., 2014; Buchon et al., 2013a; Neyen et al., 2012). Since we found that the microbiota is signaling through the Imd pathway to activate Pvf2 expression, we next tested which of the peptidoglycan recognition protein receptors (PGRPs) in this pathway was involved. We challenged the two different PGRP receptor mutants, PGRP-LE (PGRP-LE<sup>112</sup>) and PGRP-LC (PGRP-LC<sup>Δ5</sup>), along with the double mutant and observed increased DCV infection in the double mutant and PGRP-LC mutants, but not PGRP-LE mutants (Figure 6D; Figure S4B). These data suggest that peptidoglycans from the microbiota are sensed by PGRP-LC in the midgut to drive Pvf2 induction.

These data suggest that the microbiota, and in particular *A. pomorum*, is activating the Imd pathway to induce the antiviral ERK cascade. To directly test whether the antiviral activity of *A. pomorum* is dependent on the Imd pathway, we monoassociated control flies or flies mutant for the NF- $\kappa$ B gene *Rel* with *A. pomorum*. As expected, in control flies, *A. pomorum* is able to rescue antiviral function that is lost by ablation of the microbiota (Figure 6E). However, in flies mutant for *Rel*,



**Figure 4. The Microbiota Regulates Pvf2 Expression and Antiviral Defense**

(A) *Drosophila* cells were treated for 1 hr with the supernatant of the indicated bacterial species and Pvf2 expression was monitored by RT-qPCR with mean  $\pm$  SD; n = 3; \*p < 0.05.

(B) Flies fed vehicle or antibiotics were analyzed by RT-qPCR for Pvf2 mRNA normalized to rp49 and shown relative to control from 15 pooled intestines with mean  $\pm$  SD; n  $\geq$  3; \*p < 0.05.

(C) Flies raised conventionally or germ-free were analyzed by RT-qPCR for Pvf2 mRNA normalized to rp49 and shown relative to control from 15 pooled intestines with mean  $\pm$  SD; n = 3; \*p < 0.05.

(D) Representative immunoblot analysis of 20 pooled control or antibiotic-treated guts.

(E and F) Control or antibiotic-treated flies were infected with DCV (E) or VSV (F) and RT-qPCR analysis of viral RNA was normalized to rp49 and shown relative to control from 15 pooled intestines 7 dpi. Mean  $\pm$  SD; n = 4; \*p < 0.05.

(G) Conventionally reared or germ-free flies were infected with DCV and RT-qPCR analysis of DCV RNA normalized to rp49 is shown relative to control (conventionally reared flies) from 15 pooled intestines 7 dpi. Mean  $\pm$  SD; n = 6; \*p < 0.05. See also Figure S3.

*A. pomorum* is no longer protective (Figure 6E), and similar results were observed for flies mutant for Tak1 (Figure S4E). Therefore, the antiviral activity of the microbiota is dependent on Imd signaling.

Next, we tested whether virus-dependent Pvf2 induction in the intestine was NF- $\kappa$ B-dependent. We found that flies mutant for Rel were unable to induce Pvf2 upon oral viral infection (Figure 6F). We also tested the JNK pathway and found that expression of bsk<sup>DN</sup> in the intestinal epithelium had no effect on virus-induced Pvf2 levels (Figure S4F). These data suggest that activation of NF- $\kappa$ B downstream of the microbiota is required for antiviral defense in the intestine.

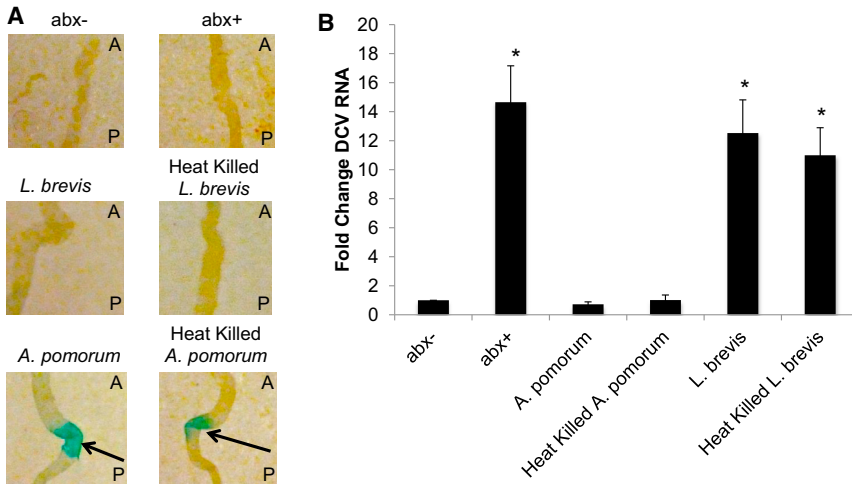
#### Virus-Induced Pvf2 Is Microbiota and Cdk9 Dependent

Altogether, these data suggest that virus-induced Pvf2 activation is downstream of the microbiota. Therefore, we tested whether we could rescue the microbiota-dependent antiviral activity by enforced Pvf2 expression. We transiently expressed Pvf2 in either conventionally reared or antibiotic-treated flies using a heat shock-inducible driver. As expected, antibiotic treatment of control flies led to increased infection, and expression of Pvf2 attenuated infection in conventional animals (Figure 7A). Furthermore, we found that ectopic Pvf2 expression is sufficient to completely restore antiviral immunity to antibiotic-treated flies

(Figure 7A). These data demonstrate that Pvf2 is downstream of the microbiota.

Moreover, these data suggest that the microbiota is required for virus-induced Pvf2 expression. To test this directly, we challenged antibiotic-treated flies with DCV and found that while conventional flies responded to viral infection by inducing Pvf2 as measured either by lacZ expression or RT-qPCR, loss of the microbiota prevented virus-induced Pvf2 expression (Figures 7B and 7C).

Therefore, these data suggest that two signals are required for Pvf2 induction. We found that the microbiota, specifically gram-negative commensals, such as *A. pomorum*, along with a virus-dependent signal are required for full activation. We previously showed that transcriptional pausing primes virally induced genes to facilitate rapid induction of the antiviral response (Xu et al., 2012). Pol II is recruited to the promoter and engages in transcriptional initiation; however, due to its association with NELF (negative elongation factor) and DSIF (DRB-sensitivity factor), Pol II can only synthesize short, abortive transcripts. Upon viral sensing, Pol II is released from pausing by recruitment of P-TEFb (positive elongation factor) leading to the rapid production of functional antiviral mRNAs (Nechaev and Adelman, 2011; Xu et al., 2012). Indeed, we previously demonstrated in cell culture that virus-induced ERK activation is pausing dependent by



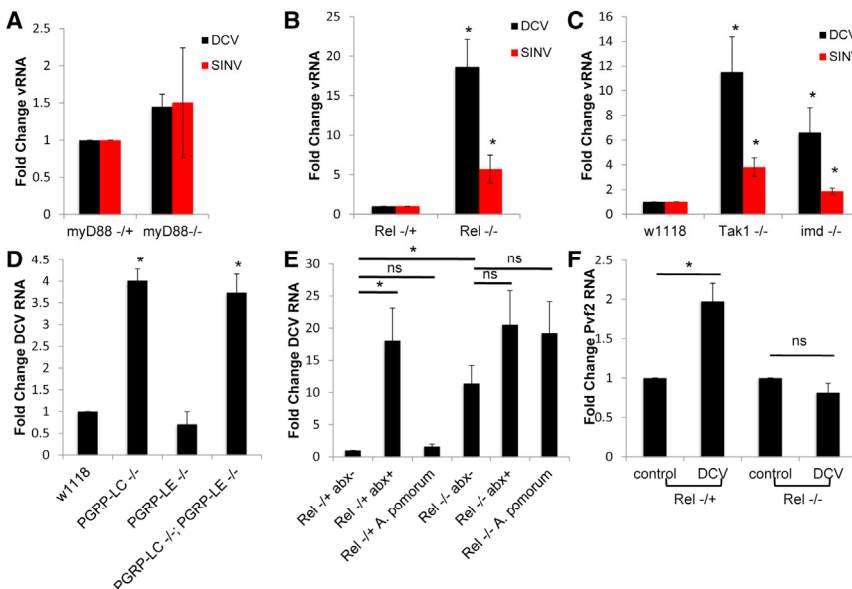
**Figure 5. *A. pomorum* Is Sufficient to Restore Antiviral Defense in the *Drosophila* Intestine**

(A) Flies carrying a Pvf2 promoter-driven lacZ reporter (Pvf2-lacZ) were associated with the indicated commensal and stained for beta-galactosidase activity. A representative image of the posterior midgut and arrows indicate induction of lacZ expression (A, anterior; P, posterior). (B) RT-qPCR analysis of DCV RNA normalized to rp49 and shown relative to control ( $w^{1118}$  abx-) from midguts of flies associated with the indicated commensal at 7 dpi. Mean  $\pm$  SD; n = 4; \*p < 0.05.

**DISCUSSION**

Enteric viruses must overcome the intestinal barrier to establish infection within the organism. Here we demonstrate that the antiviral ERK pathway is activated in the *Drosophila* intestine by the Pvf2-PVR pathway and that Pvf2 expression is induced in the posterior midgut by viral infection (Figure S5B). Additionally, the posterior midgut is the region of the gut that is most responsive to the microbiota (Broderick et al., 2014), and we found that induction of Pvf2 is dependent on microbiota signaling through the NF- $\kappa$ B pathway, which primes the antiviral response. In the absence of the microbiota, the animals are more susceptible to oral challenge and this can be overcome by ectopically expressing Pvf2 or by mono-association with *A. pomorum*, the commensal, which potently activates Pvf2, but not *L. brevis*, which does not induce Pvf2. However, the endogenous microbiota signaling through the Imd pathway is not sufficient to induce Pvf2 but requires a second signal. We found that transcriptional pausing is also required for Pvf2 induction. We previously found that transcriptional pausing is required for the induction of half of the

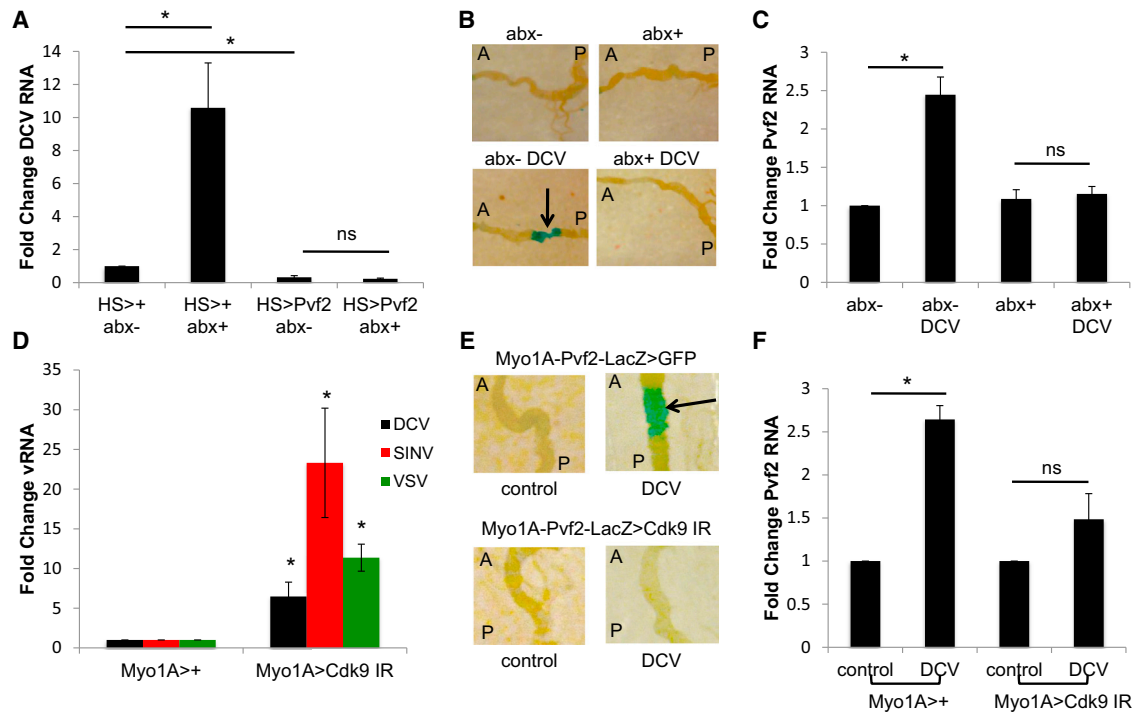
depleting Cdk9, which is a component of P-TEFb (Xu et al., 2013). Therefore, we examined the role of transcriptional pausing in oral infection. We depleted Cdk9 in the intestinal epithelium, orally challenged with DCV, SINV, or VSV, and observed a significant increase in viral infection in the intestine as measure by RT-qPCR (Figure 7D). We also used a heat shock driver to transiently deplete Cdk9 and again observed a significant increase in DCV infection (Figure S5A). Next, we examined whether transcriptional pausing is required for virus-induced Pvf2 activation using two assays. We found that virus-dependent Pvf2 expression is Cdk9 dependent both by monitoring Pvf2-lacZ expression (Figure 7E) and by measuring Pvf2 by RT-qPCR (Figure 7F). Both assays show that virus-dependent Pvf2 expression is dependent on Cdk9. Overall, we propose a model in which Pvf2 is induced by a virus-stimulated and microbiota-dependent NF- $\kappa$ B signaling cascade in the intestine, which activates epithelial antiviral ERK responses (Figure S5B).



**Figure 6. NF- $\kappa$ B Is Required for Pvf2 Expression and Antiviral Defense in the *Drosophila* Intestine**

(A-F) Flies of the indicated genotypes were infected with the indicated viruses. RT-qPCR analysis of viral (A-E) or Pvf2 (F) RNA normalized to rp49 and shown relative to control from 15 pooled intestines 7 dpi (A-E) or 4 hpi (F) with mean  $\pm$  SD; n  $\geq$  3; \*p < 0.05. See also Figure S4.





**Figure 7. Virus-Induced Pvf2 Expression Is Microbiota and Cdk9 Dependent**

(A, C, D, and F) RT-qPCR analysis of viral (A and D) or Pvf2 (C and F) RNA normalized to rp49 and shown relative to control for the indicated genotypes 7 dpi (A and D) or 4 hpi (C and F) from 15 pooled intestines. Mean  $\pm$  SD; n = 4; \*p < 0.05.

(B) Flies carrying a Pvf2 promoter-driven lacZ reporter (Pvf2-lacZ) were antibiotic treated, infected with DCV, and stained for beta-galactosidase activity at 3 dpi. A representative image of the posterior midgut and arrows indicate induction of lacZ expression (A, anterior; P, posterior).

(E) Flies of the indicated genotypes were infected with DCV stained for beta-galactosidase activity at 3 dpi. A representative image of the posterior midgut and arrows indicate induction of lacZ expression (A, anterior; P, posterior). See also Figure S5.

virus-induced genes (Xu et al., 2012), suggesting that a pausing-regulated gene is required for Pvf2 induction. Future studies will be directed toward understanding the mechanism of how transcriptional pausing cooperates with NF- $\kappa$ B to regulate Pvf2 expression.

A growing body of literature has shown that the microbiota can play a protective role in antiviral immunity against enteric viruses. Antibiotic-treated *Aedes aegypti* are more susceptible to DENV infection (Cirimotich et al., 2011; Ramirez et al., 2012; Xi et al., 2008). In mammalian intestinal cell culture, commensals were shown to block rotavirus infection (Varyukhina et al., 2012) and germ-free mice are more susceptible to coxsackievirus infection displaying an increase in virus-associated mortality (Pang and Iwasaki, 2012; Schaffer et al., 1963). Moreover, the microbiota is protective from influenza virus infection of the lung and from systemic lymphocytic choriomeningitis virus infection (Abt et al., 2012; Ichinohe et al., 2011). Our work further supports an essential role for the microbiota in maintaining the host's health defenses against viral challenges and provides mechanistic insight into molecular pathways involved.

Further, by taking advantage of the simplified *Drosophila* system, we found a role for specific members of the community. We demonstrated that the endogenous gram-negative commensals are strong inducers of the Imd pathway and therefore activate NF- $\kappa$ B signaling to induce Pvf2 expression in the epithelium. In particular, we found that *A. pomorum* is protective from viral

infection. These bacteria are sufficient to promote normal developmental time (Newell and Douglas, 2014) and for optimal growth of larvae on nutrient scarce diets (Shin et al., 2011). *A. pomorum* activates the insulin signaling pathway (Shin et al., 2011), which is known to activate ERK signaling (Kim et al., 2004; Lee et al., 2008). Therefore, *A. pomorum* may be modulating antiviral defense through two independent pathways. Interestingly, *Acetobacter* species are commonly associated with most laboratory-raised and wild-caught strains (Broderick and Lemaitre, 2012), suggesting that the protective role of *Acetobacter* could be acting in the wild.

Our finding that Pvf2 is induced in the midgut by commensals is consistent with previous studies that found that Pvf2 expression was dependent on PGRP-LC and Imd signaling (Bond and Foley, 2009). Furthermore, in addition to observing viral infection in enterocytes, we observed regional responsiveness: both virus-induced Pvf2 expression and viral infection was observed in the same region, the posterior midgut. From insects to mammals, the digestive tract is divided into distinct regions with distinct characteristics (Buchon et al., 2013b; Karasov et al., 2011). Moreover, intestinal pathologies tend to be region specific (Stainier, 2005) and therefore due to its relative simplicity and small size, the *Drosophila* intestine serves as an ideal model for examining compartmentalization and its role in disease states. Whether enteric viral infections in mammals are regionalized is unclear but likely.

Activation of NF- $\kappa$ B signaling by viral infection is commonly observed. In mosquitoes, the Imd pathway controls DENV infection in the midgut (Sim et al., 2013). Moreover, the Imd pathway activates the NF- $\kappa$ B transcription factor Rel2, which is required for antiviral defense against orally acquired viruses in the blood meal of mosquitoes (Avadhanula et al., 2009; Cirimotich et al., 2011; Paradkar et al., 2014; Xi et al., 2008). We propose that some of this regulation may be downstream of the microbiota of mosquitoes and that this regulation may require multiple inputs; NF- $\kappa$ B may be necessary but not sufficient for antiviral responses. In mammals, the intestinal epithelium senses infection of bacterial products through TLRs, including TLR2 and TLR4, which also induce NF- $\kappa$ B-dependent responses (Kumar et al., 2009; Takeuchi et al., 1999). Furthermore, TLR2 and TLR4 also activate ERK signaling (Banerjee and Gerondakis, 2007; Good et al., 2012), suggesting that there may be a functional conservation of the links between the microbiota, NF- $\kappa$ B activity, and antiviral response in the digestive tract. However, virus-intestinal interactions are clearly more complex, as in some cases enteric viruses associate with bacterial products either to stabilize them or to activate pro-viral NF- $\kappa$ B pathways (Kane et al., 2011; Kuss et al., 2011; Robinson et al., 2014). However, TLR2/4 pathways can restrict viral infection in some cases (Arpaia and Barton, 2011; Lester and Li, 2014) and thus may intersect with our findings that the microbiota influences the activation of the NF- $\kappa$ B signaling for antiviral immunity.

Recent studies have also suggested that enteric viruses can induce signals that intersect with the microbiota demonstrating unappreciated interrelationships between viral and microbiota-dependent responses (Kernbauer et al., 2014). Further, our identification of a secreted factor, Pvf2, which directly impacts enteric virus immunity in the intestine of *Drosophila* may have other parallels in mammals given the increasing appreciation of the roles of the epithelium in producing secreted factors that drive downstream immune functions (Gallo and Hooper, 2012; Peterson and Artis, 2014; Rescigno, 2011). Since we find that this microbiota-NF- $\kappa$ B-ERK pathway is active against divergent viruses, it may represent an ancient pathway that evolved to restrict infection at this mucosal surface. Since all enteric viruses come into contact with the microbiota prior to establishing infection, future studies will be directed toward understanding the mechanisms driving these interactions. Importantly, we also found that specific commensals drive different responses and a clearer understanding of how the particular commensals and the structure of the commensal population impacts immunity will inform mechanisms of dysbiosis-driven pathologies.

## EXPERIMENTAL PROCEDURES

### Fly Rearing and Infections

All fly stocks used in this study are *Wolbachia*-free and listed in Table S1. Flies were orally infected as previously described (Xu et al., 2013). In brief, 7- to 10-day-old female flies of the indicated genotypes were orally infected with 10  $\mu$ l of each virus (DCV:  $1 \times 10^{12}$  IU/ml; VSV:  $1 \times 10^9$  pfu/ml; SINV:  $1 \times 10^9$  pfu/ml; DENV-2:  $2 \times 10^8$  pfu/ml) in sucrose for 3 days and then transferred to virus-free food every 3 days or for the duration of the experiment. For survival, flies were scored daily for 20 days. Three independent replicates of 15 flies each were performed for each experiment. Heat shock flies were incubated at 37°C for 1 hr every day for 3 days prior to infection. Once orally infected, flies were incubated at 37°C for 1 hr every other day for the duration of the experiment.

### Cells and Viruses

Insect cells (DL1) were grown and maintained as described (Rose et al., 2011). VSV-GFP, SINV-GFP, DCV, and DENV-2 were grown as described (Sessions et al., 2009; Xu et al., 2012).

### Cell Culture

Amplicons used are described at <http://flyrnai.org>. dsRNA were generated and used for RNAi for 3 days as previously described (Cherry et al., 2005). For Pvf2 induction in vitro,  $3 \times 10^6$  DL1 cells were plated in a 6-well plate in complete media for 24 hr. Commensals and *E. coli* were grown and normalized to an OD of 0.1 and then 1 ml of bacteria was centrifuged at 13,000 rpm for 2 min. 30  $\mu$ l of supernatant was added to the cells for 1 hr and then processed for RT-qPCR.

### RNA and Quantitative Real-Time PCR

Total RNA was extracted from cells or 15 fly guts, using TRIzol (Invitrogen) according to manufacturer's protocol and as previously described (Xu et al., 2013). cDNA was prepared using M-MLV reverse transcriptase (Invitrogen). cDNA was analyzed using Power SYBR Green PCR Master Mix (Applied Biosystems), along with gene-specific primers in triplicate, for at least three independent experiments. Data were analyzed by relative quantification, by normalizing to rp49. Primers are listed in Table S2.

### Immunoblotting

Cells or 20 fly guts were collected and lysed with NP40 buffer supplemented with a protease inhibitor cocktail. Samples were separated and blotted as previously described for three independent experiments (Xu et al., 2013).

### X-Gal Staining

X-gal staining was performed as previously described (Choi et al., 2008). Three independent experiments were performed imaging at least five guts per condition.

### Immunofluorescence and Confocal Microscopy

Guts were processed as previously described (Xu et al., 2013). Briefly, five guts per experiment were dissected in PBS, fixed in 4% formaldehyde solution for 30 min, rinsed three times in PBS, and blocked with 5% normal donkey serum for 45 min. Samples were incubated with primary antibody (DCV capsid 1:3,000) or (Dengue 1:4,000) overnight at 4°C, rinsed 3 times in PBT, and incubated with secondary antibody (1:1,000) and Hoescht 33342 at room temperature for 1 hr 15 min. Samples were rinsed three times in PBT and mounted in Vectashield (Vector Laboratories). Guts were imaged on Leica TCS SPE confocal microscope at 10 $\times$  or 40 $\times$ . Three independent experiments were performed.

### Statistics and Data Analysis

For RT-qPCR studies, p values were obtained by comparing delta CT values for three independent experiments. For survival curves, pairwise comparisons of each experimental group with its control were carried out using a Mantel-Haenszel test. For other experiments, the Student's two-tailed t test was used to measure the statistical significance in each experiment and then considered significant if  $p < 0.05$  in each of three independent experiments.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2015.10.010>.

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