



HHS Public Access

Author manuscript

Cell Rep. Author manuscript; available in PMC 2022 January 20.

Published in final edited form as:

Cell Rep. 2021 December 28; 37(13): 110150. doi:10.1016/j.celrep.2021.110150.

Orally acquired cyclic dinucleotides drive dSTING-dependent antiviral immunity in enterocytes

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SUMMARY

Enteric pathogens overcome barrier immunity within the intestinal environment that includes the endogenous flora. The microbiota produces diverse ligands, and the full spectrum of microbial products that are sensed by the epithelium and prime protective immunity is unknown. Using *Drosophila*, we find that the gut presents a high barrier to infection, which is partially due to signals from the microbiota, as loss of the microbiota enhances oral viral infection. We report cyclic dinucleotide (CDN) feeding is sufficient to protect microbiotadeficient flies from enhanced oral infection, suggesting that bacterial-derived CDNs induce immunity. Mechanistically, we find CDN protection is dSTING- and dTBK1-dependent, leading to NF-κB-dependent gene expression. Furthermore, we identify the apical nucleoside transporter, *CNT2*, as required for oral CDN protection. Altogether, our studies define a role for bacterial products in priming immune defenses in the gut.

In brief

Segrist et al. find the cyclic dinucleotide, c-di-GMP, primes antiviral immunity in intestinal epithelial cells through the conserved apical purine transporter, *CNT2*, and the ancient dSTING-dTBK1 antiviral pathway.

Graphical abstract

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AUTHOR CONTRIBUTIONS

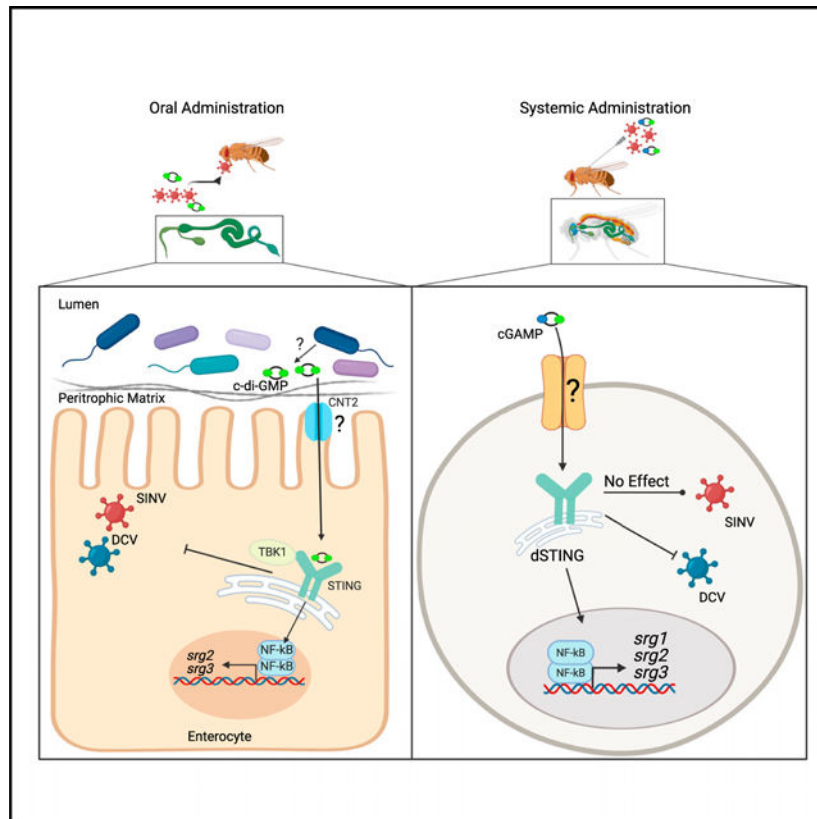
Conceptualization, E.S. and S.C.; methodology, E.S. and S.C.; investigation, E.S., B.G., and M.D.; writing, E.S. and S.C.; funding acquisitions, E.S. and S.C.; supervision, S.C.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2021.110150>.

DECLARATION OF INTERESTS

The authors declare no competing interests.



INTRODUCTION

The gut houses an extensive microbiota which influences homeostasis, nutrient uptake, and immunity. The mutualistic interaction between host and microbiome relies on the ability of microbes to colonize the gut and the host's ability to tolerate and control them. There is a vast spectrum of microbiota-derived ligands, and how they shape immunity is not fully characterized, though products like toll-like receptor (TLR) ligands and peptidoglycan prime enterocytes to promote immunity (Rakoff-Nahoum et al., 2004; Sansone et al., 2015). Commensals produce cyclic dinucleotides (CDNs) (Danilchanka and Mekalanos, 2013), but their impact on barrier immunity is unknown. *Drosophila*, with its high conservation, powerful genetic tools, and simplified biology, provides important insights into the complex interactions between host, microbiota, and enteric viral infection (Buchon et al., 2014; Xu et al., 2013).

The conserved protein STING binds endogenous CDNs produced by cGAS and exogenous bacterial CDNs and controls pathogen infections, from mammals to invertebrates (Ahn and Barber, 2019; Cai and Imler, 2021). CDNs are not cell permeable, therefore, extracellular CDNs are transported into cells. Recently, CDN transporters with tissue-specific activity were discovered (Lahey et al., 2020; Luteijn et al., 2019; Ritchie et al., 2019; Zhou et al., 2020a; Zhou et al., 2020b), and an intestinal epithelial transporter has not been identified.

In mammals, STING activation upon infection leads to interferon signaling, NF- κ B activation, and autophagy (Ahn and Barber, 2019; Cai and Imler, 2021). Less is known regarding STING sensing of commensals. Indeed, STING is implicated in intestinal homeostasis, particularly in myeloid cells (Aden et al., 2018; Ahn et al., 2017; Shmuel-Galia et al., 2021). Additionally, *Drosophila* STING (dSTING) responds to systemic sources of CDNs (Cai et al., 2020; Martin et al., 2018). However, it is unclear whether STING and CDNs play a role in antiviral immunity in the intestine.

As the breadth of antiviral pathways in enterocytes is unknown, we determined if dSTING was antiviral in the gut using the arthropod-borne virus Sindbis virus (SINV) and a picornalike virus, *Drosophila C* virus (DCV) (Johnson and Christian, 1999; Sansone et al., 2015; Xu et al., 2013). We found that dSTING is broadly antiviral in the gut and specifically active in enterocytes. We uncovered a circuit whereby orally acquired CDNs activate a dSTING-, dTBK1- dependent antiviral response that requires the apical nucleoside transporter *CNT2*. Furthermore, a CDN-STING-NF- κ B pathway leads to induction of non-canonical targets to control infection. We explored this pathway during systemic infection and found striking tissue- and virus-specific differences. Diverse CDNs, *CNT2*, and dSTING protect against oral infection of SINV and DCV, but c-di-GMP and *CNT2* do not protect against systemic infection. Moreover, dSTING and 2'3'cGAMP protect flies against systemic DCV infection but not systemic SINV infection. Therefore, our studies demonstrate exogenous CDNs activate enteric antiviral immune defenses and have more complex roles during systemic infection.

RESULTS

dSTING controls enteric virus infection

First, we determined if dSTING influences enteric immunity and observed increased virus infection in *dSTING* mutants (Figure 1A and S1A). Next, we explored which cell type (enterocytes, intestinal stem cells, or enteroendocrine cells) is required for dSTING's antiviral activity. We used tissue-specific drivers and *in vivo* RNAi to deplete dSTING in each cell type and challenged these flies with SINV. dSTING was specifically required in enterocytes for antiviral defense against SINV (Figure 1B) and DCV (Figures 1C and S1D). >90% of intestinal cells are enterocytes, so we verified dSTING depletion with an enterocyte-specific or ubiquitous driver (Figures S1B and S1C).

dSTING controls systemic Zika virus and DCV infection (Goto et al., 2018; Liu et al., 2018). Given this, we evaluated the role of dSTING during systemic SINV infection. As expected, dSTING depletion leads to increased infection of DCV in whole animals (Figure 1D) (Goto et al., 2018; Liu et al., 2018) but does not control systemic SINV infection (Figures 1A and 1D).

Mammalian STING signals through TBK1 to activate antiviral signaling pathways (Abe and Barber, 2014; Ishikawa and Barber, 2008; Watson et al., 2012). The *Drosophila* TBK1 homolog (ik2, *dTbK1*) is dispensable during *in vitro* DCV infection (Goto et al., 2018; Kuranaga et al., 2006). However, dTBK1 polymorphisms are associated with variance in suppression of bacterial infection, suggesting that it plays a role in host

defences (Lazzaro et al., 2006; Lazzaro et al., 2004). *dTBK1* is an essential gene (Shapiro and Anderson, 2006), so we used tissue-specific approaches to explore the role of *dTBK1* in enteric immunity and depleted *dTBK1* in enterocytes (Figure S1E). Next, we orally challenged these flies with SINV and observed increased viral infection (Figure 1E).

To rule out the possibility that increased infection in *dSTING*- or *dTBK1*-enterocyte-depleted flies was due to increased gut permeability, we performed a SMURF assay (Rera et al., 2012). We also performed the assay in young or aged flies, as aging increases gut permeability (Table S1) (Dambrose et al., 2016; Rera et al., 2012). However, depletion of *dSTING* or *dTBK1* did not affect gut permeability (Table S1). Since the microbiota can influence virus infection, we quantified microbiota levels and observed no change in bacterial counts in *dSTING*- or *dTBK1*-deficient flies (Table S1).

RNAi is dispensable during enteric SINV infection

The RNAi pathway is strongly antiviral during systemic viral infection, including DCV and SINV in *Drosophila* (Galiana-Arnoux et al., 2006; van Rij et al., 2006). However, RNAi is less active in certain tissues, including the brain, salivary gland, or intestine (Liu et al., 2018; Mondotte et al., 2018; Palmer et al., 2020). Indeed, *Dcr2* and *Ago2* mutants have increased systemic infection compared to controls (Figure S2A) (Liu et al., 2018). However, during oral DCV infection, *Ago2* but not *Dcr2* mutants had increased DCV RNA in the gut (Figure S2B) (Mondotte et al., 2018). In contrast, *Ago2* and *Dcr2* do not impact oral SINV infection (Figure S2B).

CDNs protect against virus infection and induce gene expression in the gut

Studies suggest that intestinal STING may sense the microbiota (Ahn et al., 2017; Shmuel-Galia et al., 2021). Therefore, we hypothesized enteric *dSTING* may be activated by CDNs produced by local commensals and thus investigated the role of orally acquired CDNs in enteric antiviral immunity. Nearly all bacteria, including human commensals, produce CDNs as essential second messengers (Corrigan and Gründling, 2013; Römling et al., 2013). Indeed, bioinformatics reveals 4 of the 7 major bacteria in the *Drosophila* microbiota encode proteins homologous to CDN synthases (Table S2) (Simm et al., 2004; Woodward et al., 2010).

To assess the role of the microbiota during SINV infection, we orally infected conventional or antibiomatic treated flies. Microbiota-depleted animals had increased SINV infection in the gut (Figure 2A). This led us to evaluate whether exogenous CDNs, potentially from the microbiota, activate *dSTING*-dependent antiviral immunity. Therefore, we explored whether feeding flies the bacterial CDN, c-di-GMP, protected them from infection. We fed c-di-GMP only to antibiomatic-treated flies to prevent any confounding effects CDNs could have on the microbiota rather than the intestine. c-di-GMP feeding protects antibiomatic-treated flies from the increase in infection caused by loss of the microbiota (Figure 2B).

Previous studies found CDN injection into the fly body cavity activates *dSTING*-dependent expression of genes, including *srg1*, *srg2*, and *srg3* (Cai et al., 2020). Thus, we determined if CDN feeding induced similar gene expression changes in the gut. *srg2* and *srg3* expression is induced by c-di-GMP feeding (Figure 2C), but *srg1* was undetectable in the gut by

qPCR (data not shown) (Dutta et al., 2015; Hung et al., 2020). We also monitored *nazo* and *dSTING*, which are induced by dSTING-NF- κ B antiviral signaling during systemic infection (Goto et al., 2018; Liu et al., 2018). c-di-GMP feeding did not change *nazo* or *dSTING* expression (Figure 2C).

We tested if the CDNs, c-di-AMP (bacteria) or 2'3' cGAMP (cGAS) and 3'3' cGAMP (cGAS/bacteria), induce expression of these genes. We confirmed antibiotic treatment ablated the microbiota (Table S3). c-di-AMP induced *srg2* expression, 3'3' cGAMP induced *dSTING*, *srg2*, and *srg3* expression, and 2'3' cGAMP induced *dSTING*, *nazo*, *srg2*, and *srg3* expression (Figures 2D–2F). In contrast, SINV did not induce expression of *srg2* and *srg3* (Figure S3A).

CDN injection protects animals against systemic DCV infection (Cai et al., 2020; Slavik et al., 2021). Similarly, co-injection of 2'3' cGAMP or c-di-AMP but not c-di-GMP protected flies from systemic DCV infection (Figure 2G). As expected, systemic 2'3' cGAMP induced *nazo*, *srg2*, and *srg3* expression (Figure 2H) (Cai et al., 2020). Surprisingly, systemic c-di-GMP induced gene expression changes despite its lack of antiviral activity against DCV (Figure 2H). Since dSTING is not antiviral systemically against INV (Figure 1A), we hypothesized exogenous CDNs would not inhibit systemic SINV infection. Indeed, we found CDNs do not protect flies from systemic SINV infection (Figures 2I and 2J).

Canonical NF- κ B signaling is not induced by oral infection or CDNs in the gut

Mammalian STING and dSTING activate NF- κ B (Goto et al., 2018; Ishikawa and Barber, 2008; Ishikawa et al., 2009; Liu et al., 2018; Martin et al., 2018; Sun et al., 2009). Therefore, we examined whether feeding SINV or c-di-GMP induces canonical NF- κ B signaling in the gut. *Drosophila* encode two NF- κ B signaling pathways, TOLL and IMD, which control expression of specific AMPs (Cornwell and Kirkpatrick, 2001; Lemaitre et al., 1996). Toll AMP expression (*Drosomyacin*) did not change, and IMD AMP expression (*Diptericin*, *Cecropin A2* [*CecA2*], and *Attacin A* [*AttaA*]) decreased after antibiotic treatment but did not change after feeding SINV or c-di-GMP (Figures S3B and S3C). Similarly, systemic CDNs do not induce expression of these AMPs (Figure S3D). In addition, SINV infection did not alter the expression of dSTING, *nazo*, or *dTBK1* (Figure S3B).

We also determined whether dSTING or dTBK1 impacted basal TOLL or IMD signaling in the intestine. *Diptericin* or *Drosomyacin* expression did not change in STING mutant, TBK1 mutant, or enterocyte-depleted intestines compared to controls (Figures S3E and S3F).

Systemic virus infection induces NF- κ B (*Relish*)-dependent expression of *dSTING* (Goto et al., 2018; Liu et al., 2018). Therefore, we monitored *dSTING* expression in NF- κ B (*Relish*) mutant flies and observed no change in *dSTING* expression in the intestine and no change in *dSTING* expression basally or during early SINV infection (Figure S3G).

dSTING is required for CDN-dependent antiviral activity

Next, we asked if the antiviral activity of orally acquired CDNs is dSTING- or dTBK1-dependent. Indeed, CDN-mediated protection from enteric SINV infection was lost in dSTING-enterocyte-depleted flies and in dTBK1-enterocyte-depleted flies compared to

control flies (Figures 3A and 3B). We confirmed antibiotic treatment ablated the microbiota (Table S3).

CDN-dependent gene expression is dSTING, dTBK1, and NF- κ B dependent

Since CDNs induce expression of *srg2* and *srg3*, we interrogated whether this was dSTING or dTBK1 dependent. Oral c-di-GMP no longer induced expression of *srg2* and *srg3* when STING was depleted in enterocytes (Figures 3C and 3D). Additionally, c-di-GMP-induced expression of *srg3* was dTBK1 dependent (Figures S3H and S3I).

Systemic CDNs induce dSTING- and NF- κ B-dependent induction of *srg2* and *srg3* expression (Cai et al., 2020). In addition, NF- κ B controls enteric viral infection of DCV and SINV (Sansone et al., 2015). Therefore, we explored whether NF- κ B is required for intestinal CDN-dependent induction of *srg2* and *srg3*. c-diGMP-dependent induction of *srg2* and *srg3* was NF- κ B dependent (Figures 3E and 3F). We confirmed antibiotic treatment ablated the microbiota (Table S3).

CNT2 is required for CDN-mediated protection from enteric infection

CDNs are charged molecules and not cell permeable (McWhirter et al., 2009). Therefore, CDNs use transporters to cross the membrane (Lahey et al., 2020; Luteijn et al., 2019; Ritchie et al., 2019; Zhou et al., 2020a; Zhou et al., 2020b). Epithelial transporters for CDNs have not been described; however, there are two conserved classes of nucleoside transporters expressed on enterocytes, namely, apical concentrative transporters (CNTs) and basolateral equilibrative transporters (ENTs) (Sankar et al., 2002). Single-cell RNA-seq studies of the *Drosophila* intestine revealed expression of *cnt2*, *ent1*, and *ent2* but not *cnt1* (Figures 4A and S4A) (Dutta et al., 2015; Hung et al., 2020). We hypothesized that the apical *CNT2* would be more likely to interact with CDNs than the basolateral ENTs, as they are not exposed to intestinal contents. Nevertheless, we tested the role of the three conserved transporters expressed in enterocytes in antiviral immunity. We verified efficient depletion of the transporters in the gut (Figure S4B). ENT1 and ENT2 were not antiviral against SINV (Figure 4B). In contrast, CNT2 enterocyte depletion increased enteric SINV and DCV infection (Figures 4B and 4C). Importantly, loss of CNT2 in enterocytes did not impact gut permeability or microbiota loads (Table S1). Since CDNs are antiviral against DCV during systemic infection, and this is dSTING dependent (Cai et al., 2020), we tested whether CNT2 controls systemic DCV or SINV infection and found it did not (Figure 4D). This suggests that CNT2 plays an enterocyte-specific antiviral role.

CNT2 preferentially transports purines (Ritzel et al., 1998), but it is unknown whether CNT2 transports purine containing CDNs. Therefore, we tested if CNT2 is required for CDN-dependent antiviral activity in the gut. We confirmed ablation of the microbiota (Table S3). Indeed, CDN-dependent antiviral activity was CNT2 dependent (Figure 4E).

To rule out the possibility that oral c-di-GMP is degraded to guanosine, a known CNT2 ligand, and is antiviral through a purine salvage pathway, we fed antibiotic-treated control flies guanosine and orally infected them with SINV. We confirmed ablation of the microbiota (Table S3). Unlike CDNs, guanosine feeding did not protect flies from SINV infection (Figure 4F) and does not induce expression of *srg2* in the intestine (Figure 4G).

DISCUSSION

Altogether, we defined a circuit whereby exogenous CDNs and dSTING play a fundamental role in antiviral defense of enterocytes to diverse viruses. CDN-dependent antiviral activity requires the apical nucleoside transporter CNT2. Intracellularly, this CDN pathway requires both dSTING and dTBK1, which has not previously been implicated in immune defense in flies (Goto et al., 2018). Moreover, enteric CDNs induces gene expression changes in enterocytes, which are dSTING-, dTBK1-, and NF- κ B-dependent. Canonical NF- κ B genes are not induced by CDN stimulation, which indicates that CDNs induce a distinct, non-canonical gene program. Future studies will define the activities of these CDN-induced genes in viral infection.

There are striking differences in requirements for the CDN-STING pathway between enteric and systemic infection. For example, dSTING and dCNT2 are antiviral during oral SINV infection of the intestine but not during systemic infection. Also, c-diGMP protects the intestine from oral but not systemic virus infection. Moreover, the canonical insect antiviral pathway, RNAi, has minimal influence on enteric virus infection despite being essential during systemic infection. This work highlights the importance of CDN-STING signaling, the breadth of enteric antiviral pathways, and virus- and tissue-specific differences in antiviral signaling.

Also, distinct CDNs led to overlapping gene expression changes and distinct antiviral activity in the same tissue. This may be due to differences between CDN binding strength to dSTING and subsequent variation in strength of STING activation or suggests the presence of negative regulators of CDNs, or activation of STING in different tissues may be due to the expression of different transporters with distinct specificities for these CDNs. For example, known CDN transporters display tissue-specific activity and varying ability to transport different CDNs (Lahey et al., 2020; Luteijn et al., 2019; Ritchie et al., 2019; Zhou et al., 2020a; Zhou et al., 2020b).

Our discovery that exogenous CDNs impact immunity has parallels in mammalian systems and has broad implications in enteric biology (Aden et al., 2018; Ahn et al., 2017; Shmuel-Galia et al., 2021). First, CDN recognition may be deeply conserved, as it is likely commensals from flies to humans produce CDNs. Second, microbiota shifts and dysbiosis influence immunity and health. This may be partially due to changes in the presence, type, amount, and location of CDNs in the intestine, which would subsequently impact STING activity, consequently altering the threshold for inflammatory responses and innate immune protection. Future studies will undoubtedly explore how changes in levels of commensal-derived CDNs contribute to disease.

Limitations of the Study

We suggest that the *Drosophila* microbiota produces CDNs as nearly all bacteria synthesize CDNs and some species of the *Drosophila* microbiota have CDN synthase homologs, but further mediated protection from infection. This interaction is not due work must be performed to confirm these findings. Additionally, to c-di-GMP degradation into the CNT2 ligand guanosine, which CNT2 is antiviral in the intestine and is required for c-di-GMP-

leads us to hypothesize that CNT2 acts as a c-di-GMP transporter on enterocytes. However, in vitro and biochemical work needs to verify if *CNT2* is a bona fide CDN transporter.

STAR★METHODS

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be direct to and will be fulfilled by the lead contact, Sara Cherry (cherrys@penncmedicine.upenn.edu).

Materials Availability—This study did not generate new unique reagents.

Data and Code Availability

- This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resource table. All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila melanogaster—All fly stocks used are Wolbachia free and listed in the key resource table. Flies were maintained on standard cornmeal food at room temperature. 4–7 day old female flies of the indicated genotype were used. Only female flies were used as we use flies to study mosquito intestinal biology, and only female mosquitoes take blood meals and are enterically infected with arboviruses. Flies used in this paper had never previously been experimented on. Siblings were randomly sorted into experimental groups.

Cell culture—DL1 cells (sex unknown) were cultured in Schneider's medium (Invitrogen-GIBCO, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 U/mL of penicillin, and 100 mg/mL streptomycin (Invitrogen-GIBCO, Carlsbad, CA) (Rose et al., 2011) at 25°C.

Virus generation—Sindbis virus (HRsp) was generated in BHK cells and propagated in C6/36 cells (Burnham et al., 2007; Rose et al., 2011) at 37°C. DCV was propagated by infecting *Drosophila* cells maintained at 25°C and collecting cells and supernatant after 75% of cells had died (Cherry and Perrimon, 2004; Xu et al., 2012). Infected cells and supernatant were then subject to ultracentrifugation steps and resuspended in PBS.

METHOD DETAILS

Drosophila genetics and infection—For antibiotic experiments flies were fed an antibiotic cocktail (Doxycycline 640 µg/mL, Ampicillin 640 µg/mL, Kanamycin 1 mg/mL, and Rifampicin 200 µg/mL) for × days on standard cornmeal food.

Oral infections were performed as previously described (Sansone et al., 2015; Xu et al., 2013). In brief, flies were starved and infected on cornmeal food (SINV, 150 μ l, 2.1×10^9 pfu/mL) or whatman (DCV, 10 μ l, 5.17×10^7 pfu/mL) supplemented with sucrose (4% final solution). Flies of the indicated genotype were infected with SINV and placed onto new food with virus \times days later or were infected with DCV and flipped onto fresh, virus-free whatman \times days later. At 6dpi, intestines (n = 15) were collected and pooled for RT-qPCR.

For systemic injections flies of the stated genotypes were inoculated with 50nL of DCV using an Eppendorf Femtojet (Cherry and Perrimon, 2004; Liu et al., 2018; Moy et al., 2014). For CDN systemic administration 50 nL of a 1 mg/mL CDN solution (PBS) or PBS only was injected intrathoracically into flies of the indicated genotype. The indicated number of independent experiments with pools of 5 flies were used for all RT-qPCR experiments.

For CDN and guanosine feeding experiments flies were antibiotic treated, starved, and transferred onto whatman with virus and c-di-GMP (200 μ g/mL, endotoxin free, Invivogen #tlrl-nacdg) or guanosine (200 μ g/mL, for use in cell culture, Milipore Sigma, #G6264) and infected as above. c-di-GMP or guanosine were replaced three days later with virus and intestines (n = 15) were collected at 6dpi. For gene expression studies after CDN or guanosine feeding, flies were mock or antibiotic treated for three days. Then starved for one h and transferred to whatman with PBS, mock (sterile water, sterile water and DMSO), c-di-GMP (sterile water), or guanosine (sterile water and DMSO), and sucrose (4%). At 6 h after feeding, 15 intestines were dissected for RT-qPCR and 5 intestines were dissected for plating. The indicated number of independent experiments were used for all RT-qPCR experiments.

TCID50—Flies were orally infected with DCV and 30 intestines were dissected and pooled in 100 μ L Schneider's media at 7dpi. Fly intestines were homogenized and centrifuged (13,000 rpm, 10 min) to separate tissue from supernatant. Supernatants were serially diluted onto DL1 cells (5×10^4) in a 96 well format. At 48 hpi, DL1 cells were fixed and stained with anti-DCV capsid antibody (1:1000, (Cherry and Perrimon, 2004)). The Reed Muench method was used to determine TCID50, samples were normalized by the gut numbers in each sample. The end point dilution method was used to determine the limit of detection as previously described (Chan et al., 2009). We normalized the log transformed TCID50/mL LOD value to TCID50/mL/gut to directly compare the LOD to experimental TCID50/mL/gut values. The LOD was $0.872 \text{ Log}_{10} \text{TCID50/mL/gut}$.

RNA extraction and qRT-PCR—Total RNA was extracted from 15 fly intestines or 5 whole flies using TRIzol (Invitrogen), DNase 1 treatment, and the RNA Clean and Concentrator Kit (Zymo Research) according to the manufacturers' protocol. The SuperScript III cDNA synthesis kit (Invitrogen) was used to generate cDNA. cDNA was analyzed in triplicate with gene specific primers and SYBR Green PCR Master Mix (Applied Biosystems) for at least three independent experiments. RT-qPCR was used to quantify viral RNA levels or RNA levels of the genes indicated. All data was normalized to rp49 by relative quantification using the ddCT method. Primers are listed in Table S2.

Western blotting—5 animals were homogenized in protein lysis buffer consisting of 1x radioimmunoprecipitation assay (RIPA) buffer, Protease Inhibitor cocktail (cOmplete Protease Inhibitor Cocktail, Sigma Aldrich), and Phenylmethylsulfonyl fluoride (Sigma Aldrich). Protein samples were mixed with SDS sample buffer (Sigma-Aldrich) containing 5% beta-mercaptoethanol and boiled for 10 min. Samples were chilled on ice for two minutes and ran on a 10% SDS-PAGE gel (Bio-rad) and transferred to a PVDF membrane (EMD Milipore). Anti-beta Actin (1:1000, Santa Cruz #4778) and anti-dmSTING (1:500, (Martin et al., 2018)) antibodies were incubated with the membrane overnight at 4°. The membranes were then incubated with anti-ms-HRP or anti-Rb-HRP antibody respectively (1:10000, Fisher Scientific (mouse), Sigma-Aldrich (rabbit) and visualized with ECL (Amersham).

Bacterial plating and quantification—5 intestines were dissected in sterile PBS in a sterile 6-well plate and homogenized in 100 µL of a 50% glycerol-water solution. Serial dilutions were plated onto MRS plates (10µl) and were allowed to grow at 29°C for two days. CFU/mL/gut or CFU/mL/Fly was calculated.

Smurf assay—The smurf assay was performed as previously described (Dambrose et al., 2016; Rera et al., 2012). In summary, flies were fed FD&C Blue Dye #1 on whatman and scored as Smurf+ or Smurf-. The proportion of Smurf+ flies was calculated by dividing the number of Smurf+ flies by the total number of flies scored for the indicated genotype. For the aging experiment, 63–67 day old flies were used.

QUANTIFICATION AND STATISTICAL ANALYSIS

P values for RT-qPCR experiments were obtained by performing a two-tailed t test or one-way ANOVA with multiple comparisons and correction for multiple tests on ddCT values from at least three independent experiments. Visualization of data was performed in Prism 8 (Graphpad). The statistical parameters for experiments can be found in the figure legends, n indicates independent experiments. The number of animals used per experiment can be found in the method details section. Significance was defined as $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We would like to thank Bloomington, VDRC, E. Baehrecke, N. Silverman, N. Perrimon, and Excelix for fly stocks. We would like to thank R. Hardy for SINV (hrSp) and A. Goodman for dmSTING antibody. We would like to thank the lab of S.C. for helpful discussions and advice. Biorender was used for the graphical abstract. This work was supported by NIH T32-AI-007324 and NIH F31 AI147415–01 to E.S. and R01AI152362, R01AI122749, R01AI150246, and R01AI140539 to S.C. S.C. is a recipient of the Burroughs Wellcome Investigators in the Pathogenesis of Infectious Disease Award.

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Highlights

- Cyclic dinucleotides (CDNs) are sensed by enterocytes to induce antiviral protection
- CDNs signal through dSTING and NF- κ B to induce gene expression
- Antiviral activity of CDNs is dependent on the apical nucleoside transporter CNT2
- Antiviral activities of CDNs and dSTING are tissue specific

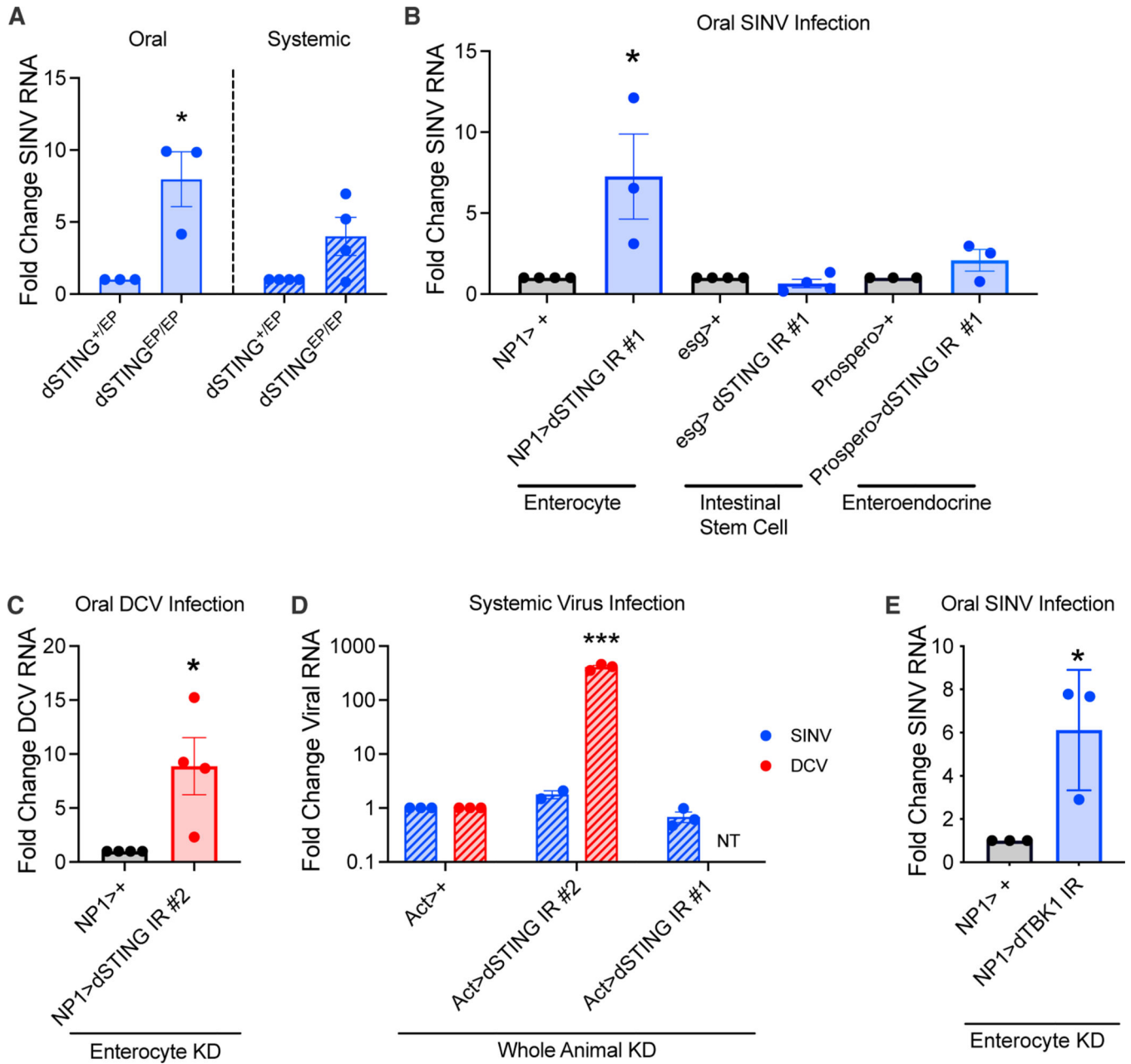


Figure 1. The dSTING-dTBK1 pathway is antiviral in the intestine

(A) Control (*dSTING^{EP/+}*) and *dSTING* mutant (*dSTING^{EP/EP}*) flies were orally or systemically infected with SINV. n = 3.

(B) Flies with *STING* depleted in enterocytes (*NP1>dSTING IR #1*), intestinal stem cells (*esg>dSTING IR #1*), and enteroendocrine cells (*Prospero>dSTING IR #1*) and their controls (*NP1>+*, *esg>+*, or *Prospero>+*) were orally infected with SINV. n = 3–4.

(C) Control (*NP1>+*) and *dSTING*-enterocyte-depleted (*NP1>dSTING IR #2*) flies were orally infected with DCV. n = 4.

(D) Control (*Act>+*) or *dSTING*-depleted (*Act>dSTING IR #1, #2*) flies were systemically infected with SINV or DCV. NT indicates not tested. n = 2–3.

(E) Control (*NPI>+*) or dTBK1-enterocyte-depleted (*NPI>dTBK1* IR) flies were orally infected with SINV. n = 3.

(A–E) qRT-PCR was performed to quantify viral RNA levels. Each dot represents an independent replicate with bars indicating mean \pm SEM. Statistical significance was determined by a Student's t test; * denotes $p < 0.05$; *** denotes $p < 0.001$. See also Figures S1 and S2 and Tables S1 and S3.

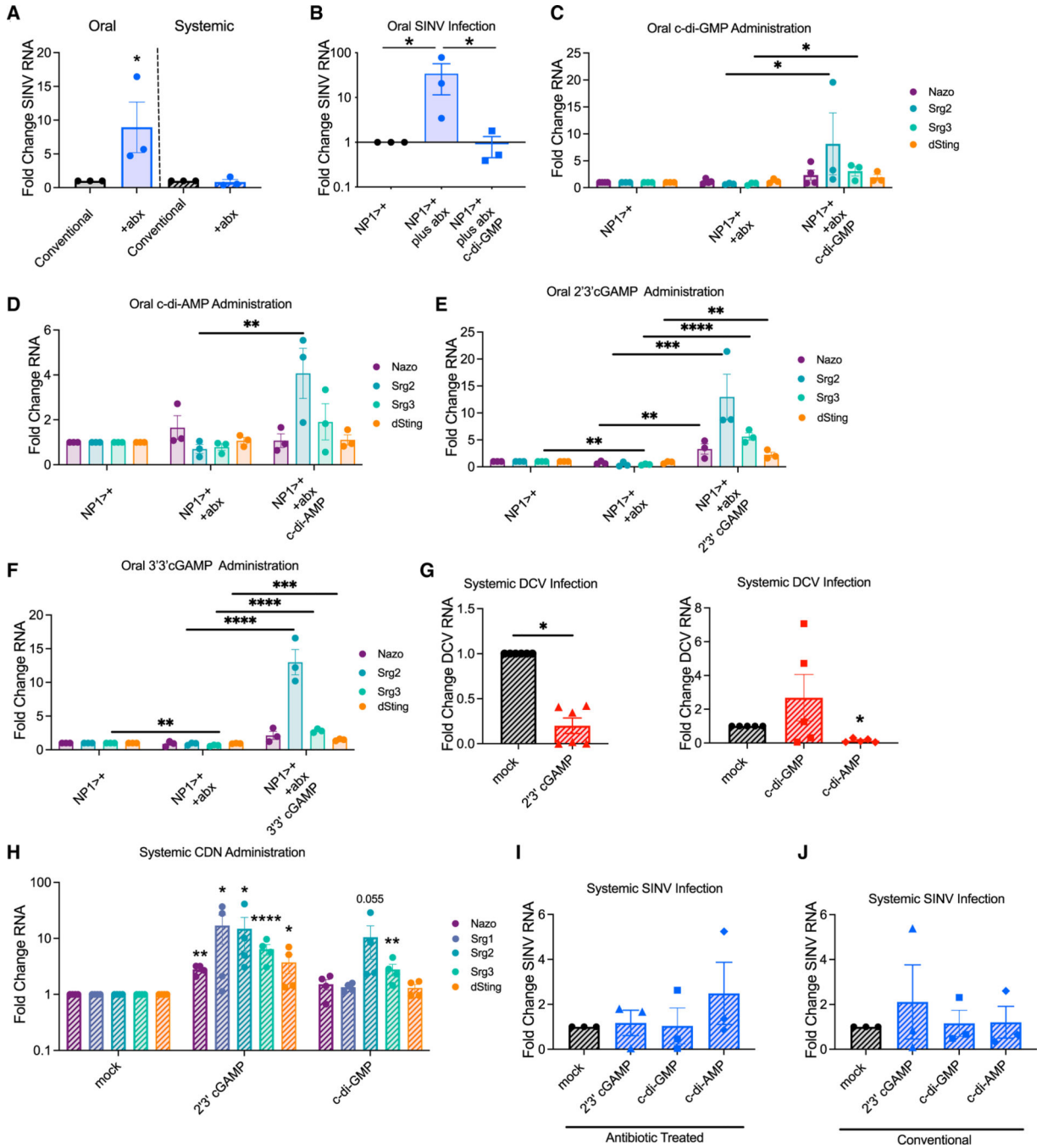


Figure 2. Exogenous CDNs are antiviral and induce gene expression changes

(A) Conventional and antibiotics-treated control flies (*w1118*) were orally or systemically infected with SINV. n = 3.

(B) Conventional and antibiotics-treated control flies (*NPI>+*) were mock or c-di-GMP fed and infected orally with SINV. n = 3.

(C–F) Conventional and antibiotics-treated control flies (*NPI>+*) were mock or (C) c-di-GMP, (D) c-di-AMP, (E) 2'3'cGAMP, or (F) 3'3'cGAMP fed. n = 3–4.

(G) Control flies (*w1118*) were co-injected with DCV and mock, 2'3'cGAMP, c-di-GMP, or c-di-AMP.

(H) Control flies (*w1118*) were injected with mock, 2'3' cGAMP, or c-di-GMP. n = 4.

(I and J) (I) Antibiotic-treated and (J) conventional control flies (*w1118*) were co-injected with SINV and mock, 2'3'cGAMP, c-di-GMP, or c-di-AMP. n = 3.

(A–J) qRT-PCR was performed to quantify viral RNA or RNA of indicated genes.

(A and G) A Student's t test was performed to determine statistical significance.

(B–F and G –J) A one-way ANOVA with multiple comparisons was performed to determine statistical significance.

(A–J) Each dot represents an independent replicate with bars indicating mean \pm SEM. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$; **** denotes $p < 0.0001$. See also Figure S3 and Tables S2 and S3.

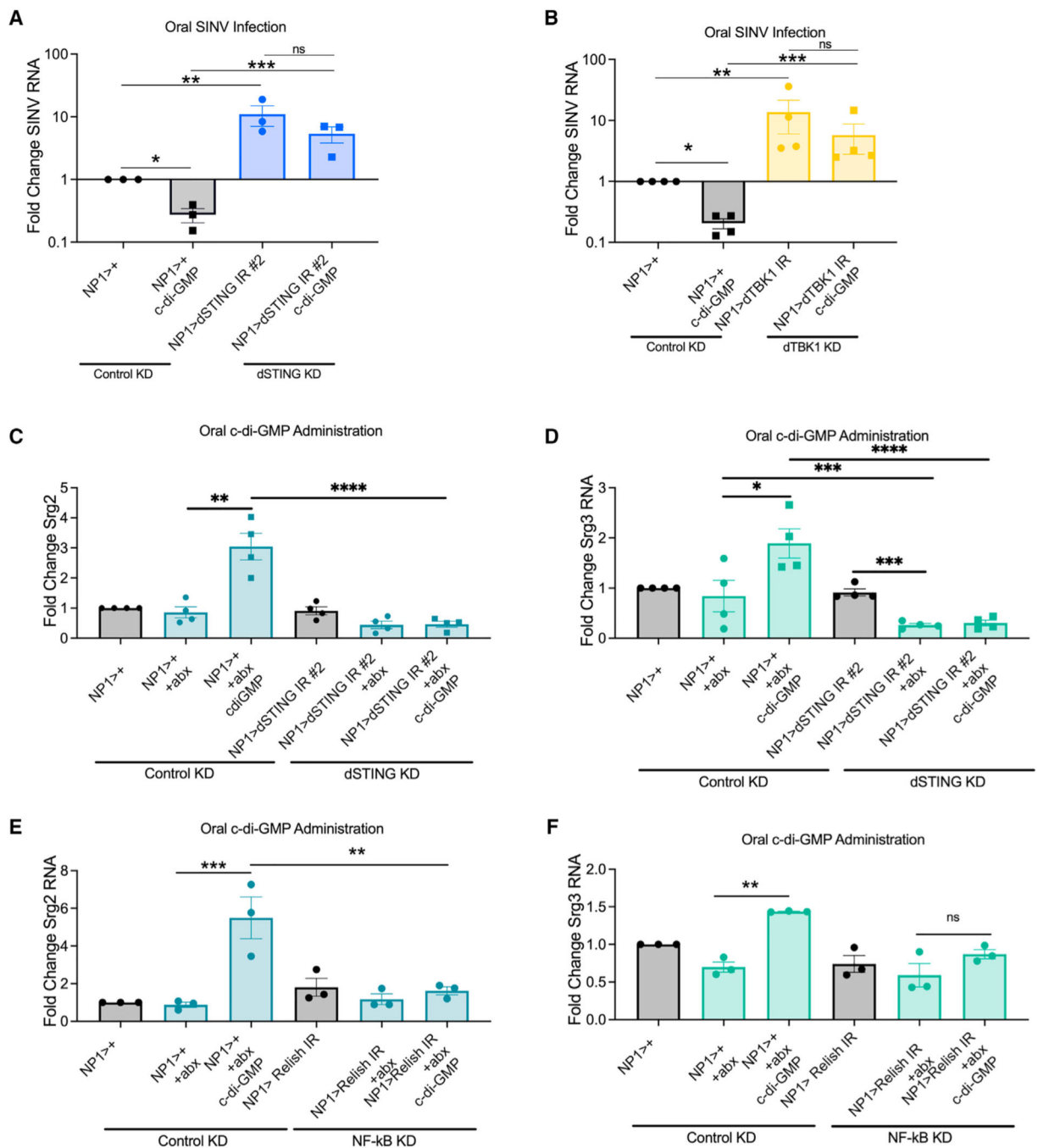


Figure 3. dSTING and Relish are required for CDN-induced gene expression or CDN-mediated protection against viral infection

(A and B) (A) Control (*NP1>+*) and dSTING-enterocyte-depleted flies (*NP1>dSTING IR #2*) and (B) dTBK1-enterocyte-depleted flies (*NP1>dTBK1 IR*) were antibiotic treated, mock or c-di-GMP fed, and orally infected with SINV. qRT-PCR was performed to quantify viral RNA levels. n = 3–4.

(C–F) Conventional and antibiotic-treated control (*NP1>+*) and (C and D) dSTING-enterocyte-depleted flies (*NP1>dSTING IR #2*) and (E and F) NF-kB- (*Relish*) enterocyte-

depleted flies (*NPI>Relish* IR) were mock or c-di-GMP fed. qRT-PCR was performed to quantify (C and E) *srg2* and (D and F) *srg3* RNA levels. n = 3–4.

(A–F) Each dot represents an independent replicate with bars indicating mean \pm SEM.

A one-way ANOVA with multiple comparisons was performed to determine statistical significance. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$; **** denotes $p < 0.0001$. See also Figure S3 and Table S3.

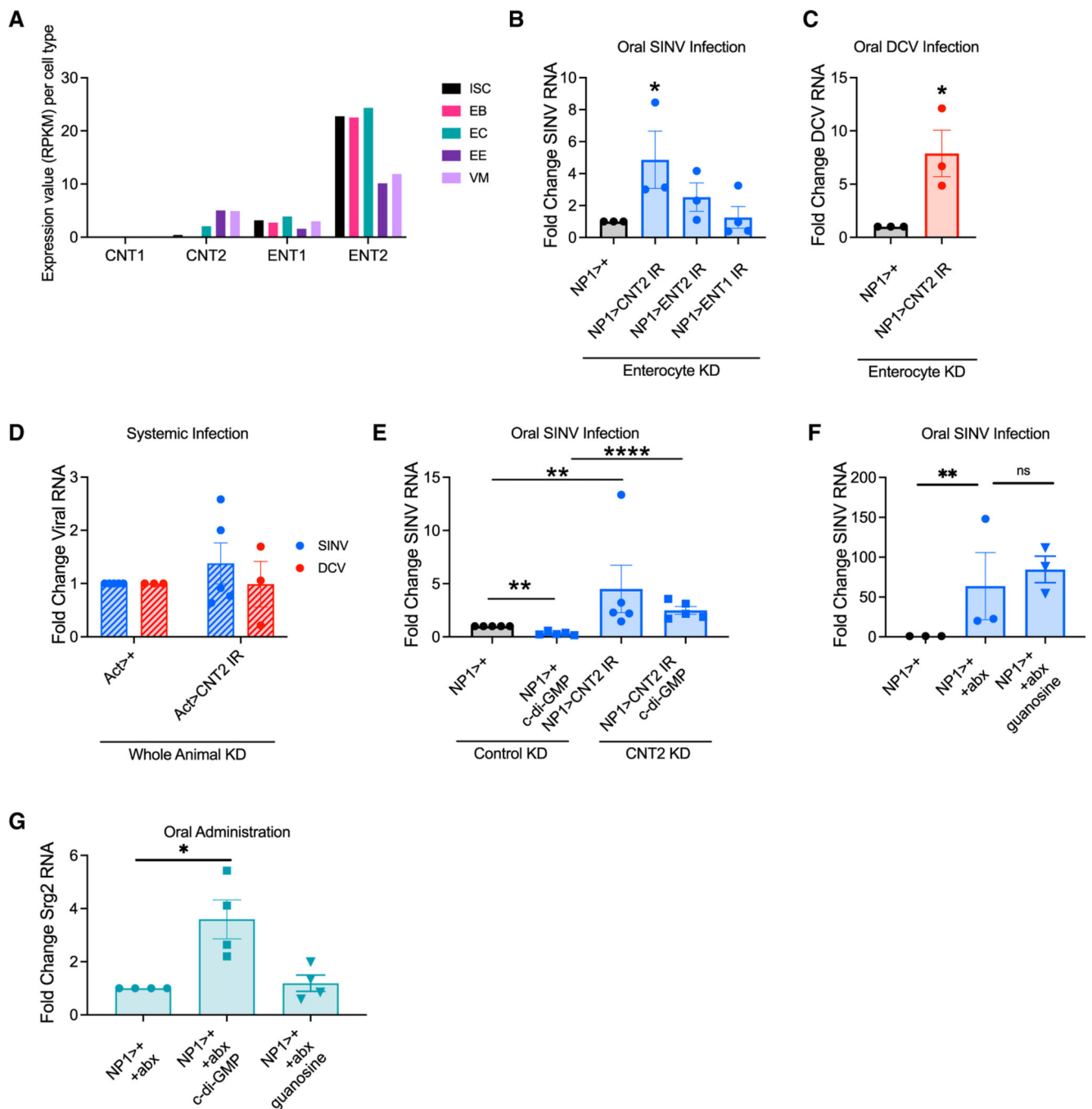


Figure 4. CNT2 is required for CDN-dependent enteric defense

(A) Reanalysis of published single-cell RNA-seq expression data for *cnt1*, *cnt2*, *ent1*, and *ent2* across cell types in the *Drosophila* intestine (Dutta et al., 2015).

(B) Control (*NP1>+*) or CNT2-, ENT2-, or ENT1-enterocyte-depleted (*NP1>cnt2 IR*, *NP1>ent2 IR*, *NP1>ent1 IR*) flies were orally infected with SINV. n = 3–4.

(C) Control (*NP1>+*) or CNT2-enterocyte-depleted (*NP1>cnt2 IR*) flies were orally infected with DCV. n = 3.

(D) Control (*Act>+*) and CNT2-depleted animals (*Act>cnt2*IR) were systemically infected with SINV or DCV. n = 3–4.

(B–D) A Student's t test was performed to determine statistical significance.

(E) Control (*NPI>+*) and CNT2-enterocyte-depleted (*NPI>cnt2*IR) flies were antibiotic treated, mock or c-di-GMP fed, and orally infected with SINV. n = 5.

(F) Conventional or antibiotic-treated control (*NPI>+*) flies were mock or guanosine fed and orally infected with SINV. n = 3.

(B–F) qRT-PCR was performed to quantify viral RNA levels.

(G) Antibiotic-treated control (*NPI>+*) flies were fed vehicle, c-di-GMP, or guanosine.

qRT-PCR was performed to quantify srg2 RNA levels. n = 4.

(E–G) A one-way ANOVA with multiple comparisons was performed to determine statistical significance.

(A–G) Each dot represents an independent experiment with bars indicating mean \pm SEM. * denotes $p < 0.05$; ** denotes $p < 0.01$; **** denotes $p < 0.0001$. See also Figure S4 and Tables S1 and S3.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Beta-Actin	Santa Cruz Technology	Cat#4778; RRID:AB_626632
dmSTING	(Martin et al., 2018)	N/A
DCV capsid	(Cherry and Perrimon, 2004)	N/A
anti-chicken-Alexa 488	Fisher Scientific	Cat# A-11039; RRID:AB_142924
anti-mouse IgG-HRP	Fisher Scientific	Cat#31430; RRIS: AB_228307
anti-Rabbit- IgG-HRP	GE Healthcare	Cat# NA934; RRID:AB_772206
Bacterial and virus strains		
SINV (HRsp)	Richard W. Hardy (Burnham et al., 2007)	N/A
DCV	Peter D. Christian (Johnson and Christian, 1999)	N/A
Chemicals, peptides, and recombinant proteins		
FD&C Blue Dye #1	Spectrum Chemical	Cat#FD110-25GM; CAS# 3844-45-9
c-di-GMP	Invivogen	Cat#turl-nacdg; CAS# 61093-23-0
c-di-AMP	Invivogen	Cat#turl-nacda; CAS# 54447-84-6
3'3'cGAMP	Invivogen	Cat#turl-nacga; CAS#849214-04-6
2'3'cGAMP	Invivogen	Cat#turl-nacga23-1; CAS# 1441190-66-4
guanosine	Millipore Sigma	Cat#G6264; CAS#118-00-3
Critical commercial assays		
SuperScript III cDNA synthesis kit	Invivogen	Cat#18080044
SYBR Green PCR Master Mix	Applied Biosystems	Cat#4367659
Deposited data		
Single cell RNA-seq data of <i>Drosophila</i> intestine	(Dutta et al., 2015)	GEO. GSE120537; https://doi.org/10.1016/j.celrep.2015.06.009
Single cell RNA-seq data of <i>Drosophila</i> intestine	(Hung et al., 2020)	GEO. GSE120537; https://doi.org/10.1073/pnas.1916820117
Experimental models: Cell lines		
<i>D. melanogaster</i> : DL1	Norbert Perrimon (Cherry and Perrimon, 2004)	N/A
<i>Aedes albopictus</i> : C6/36	ATCC	ATCC.CRL-1660; RRID: CVCL Z230
Experimental models: Organisms and strains		
<i>D. melanogaster</i> : w1118	N. Perrimon (Harvard Medical School, Boston, MA)	N/A
<i>D. melanogaster</i> : 5905	Bloomington Drosophila Stock Center	FBal0018186; RRID.BDSC_5905

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>D. melanogaster</i> : Myo1A-Gal4	E. Baehrecke (University of Massachusetts Medical School, Worcester, MA)	FBtp0098092
<i>D. melanogaster</i> : Act-Gal4	Bloomington Drosophila Stock Center	FBst0004414; RRID:BDSC_4414
<i>D. melanogaster</i> : Escargot-Gal4	N. Perrimon (Harvard Medical School, Boston, MA)	N/A
<i>D. melanogaster</i> : Prospero-Gal4	Bloomington Drosophila Stock Center	FBst080572. RRID:BDSC_80572
<i>D. melanogaster</i> : UAS-ik2 IR	Bloomington Drosophila Stock Center	FBst0034709; RRID:BDSC_34709
<i>D. melanogaster</i> : dSTING [EY0649]	Bloomington Drosophila Stock Center	BDSC.16729; RRID:BDSC_16729 Cell Reports 37, 110150, December 28, 2021 e1
<i>D. melanogaster</i> : UAS-STING IR (TRiP. JFO1138) (STING #1)	Bloomington Drosophila Stock Center	BDSC:31565; RRID:BDSC_31565
<i>D. melanogaster</i> : UAS-STING IR (GD1905) (STING #2)	Vienna Drosophila Resource Center	RRID:Flybase_FBst0463487; Cat#VDRC:4031
<i>D. melanogaster</i> : UAS-CNT2 IR (KK100597)	Vienna Drosophila Resource Center	RRID:Flybase_FBst0477654; Cat#VDRC:105828
<i>D. melanogaster</i> : UAS-ENT2 IR (KK104419)	Vienna Drosophila Resource Center	RRID:Flybase_FBst04272337; Cat#VDRC:100464
<i>D. melanogaster</i> : UAS- ENT1 IR (GD783)	Vienna Drosophila Resource Center	RRID:Flybase_FBst0468399; Cat#VDRC :49328
<i>D. melanogaster</i> : UAS-Relish IR (TRiP.HMS00070)	Bloomington Drosophila Stock Center	FBst0033661; RRID: BDSC_33661
<i>D. melanogaster</i> : AGO2 ⁴¹⁴	Bloomington Drosophila Stock Center	FBst0313641
<i>D. melanogaster</i> : Dcr-2 ^{L811fsX}	Bloomington Drosophila Stock Center	FBst0033053; RRID:BDSC_33053
Oligonucleotides		
See Table S4		N/A
Software and Algorithms		
Graphpad Prism 8	Graphpad	https://www.graphpad.com