2'3'-cGAMP triggers a STING and NF-κB dependent broad antiviral response in Drosophila

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Abstract

We previously reported that an orthologue of STING regulates infection by picorna-like viruses in drosophila. In mammals, STING is activated by the cyclic dinucleotide 2'3'-cGAMP produced by cGAS, which acts as a receptor for cytosolic DNA. Here, we show that injection of flies with 2'3'-cGAMP can induce expression of dSTING-regulated genes. Co-injection of 2'3'-cGAMP with a panel of RNA or DNA viruses results in significant reduction of viral replication. This 2'3'-cGAMP-mediated protection is still observed in flies mutant for the genes *Atg7* and *AGO2*, which encode key components of the autophagy and small interfering RNA pathways, respectively. By contrast, it is abrogated in flies mutant for the NF-κB transcription factor Relish. Analysis of the transcriptome of 2'3'-cGAMP injected flies reveals a complex pattern of response, with early and late induced genes. Our results reveal that dSTING regulates an NF-κB -dependent antiviral program, which predates the emergence of interferons in vertebrates.

INTRODUCTION

Insects, like all animals, are plagued by viral infections, which they oppose through their innate immune system. Induced transcription of antiviral genes upon sensing of infection is a common antiviral response observed across kingdoms. In insects, inducible responses contribute to defense against viruses, together with RNA interference (RNAi) and constitutively expressed restriction factors (reviewed in [1]). Apart from RNAi, these mechanisms are still poorly characterized and appear to be largely virus-specific[2-4]. Combining genetics and transcriptomic analysis, we previously showed that the evolutionarily conserved factor drosophila Stimulator of InterferoN Genes (dSTING) participates together with the kinase IKKβ and the NF-κB transcription factor Relish in a novel pathway controlling infection by the picorna-like viruses Drosophila C virus (DCV) and Cricket Paralysis Virus (CrPV) in the model organism *Drosophila melanogaster*[5].

In mammals, STING is a central component of the mammalian cytosolic DNA sensing pathway, where it acts downstream of the receptor cyclic GMP-AMP synthase (cGAS)[6]. Upon binding DNA, cGAS synthesizes 2'3'-cGAMP, a cyclic dinucleotide (CDN) secondary messenger that binds to and activates STING[7-14]. Bacteria also synthesize CDNs such as c-di-AMP, c-di-GMP and 3'3'-cGAMP, which can be sensed by STING (reviewed in [15]). Upon activation, STING recruits through its C-terminal tail (CTT) region the kinase TBK1, which phosphorylates and activates the transcription factor Interferon Regulatory Factor (IRF) 3 to trigger interferon (IFN) production[16-18]. STING can also activate NF-κB and autophagy independently from its CTT domain in mammalian cells[19-21].

The identification of STING in animals devoid of interferons, such as insects, raises the question of the ancestral function of this molecule. Invertebrate STING lacks the CTT extension, which was shown to be essential for the activation of IRF transcription factors and induction of interferons[22]. In contrast, the ability of STING to regulate transcription factors of the NF-κB family[5,23,24] or autophagy[25], seems conserved throughout metazoa. Importantly, these responses are triggered in a CTT-independent manner in mammals[19-21]. Apart from the missing CTT, the global overall structure of STING is well conserved between vertebrates and invertebrates. Accordingly, in vitro studies with STING recombinant proteins from the sea anemone *Nematostella vectensis* (Cnidaria), the oyster *Crassostrea gigas* (Mollusks) and the worm *Capitella teleta* (Annelids) revealed that they all bind CDNs[26]. Intriguingly however, binding of CDNs was not observed with recombinant STING produced from several insect species, including drosophila[26].

The mechanism by which STING exerts its antiviral effect in insects, which could provide important clues on its ancestral function, is still unclear. Here, we identify 2'3'-cGAMP as a potent agonist of dSTING in vivo and show that it triggers a strong Relish-dependent transcriptional response that confers protection against a broad range of RNA and DNA viruses.

RESULTS

A subset of CDNs trigger expression of STING dependent virus regulated genes

To characterize in vivo the dSTING pathway, we used *dSTING*^{Rxn} (RXN) loss of function mutant flies (fig. S1A). Expression of *dSTING* was reduced by 9- to 27-fold in the mutant, as previously described, but was restored to wild type level when a genomic rescue was introduced in the flies (fig. S1B). Basal levels or induction by DCV of three previously described IKKβ and dSTING dependent genes (CG13641, CG42825, and CG33926, hereafter referred to as *sting regulated gene* (*srg)1*, *srg2* and *srg3*, respectively) was significantly reduced in RXN mutant flies compared to *dSTING*^{Control} (WT) or *dSTING*^{Rescue} flies (fig. S1C-E). By contrast, induction of the gene *Hsp26*[27] by DCV (fig. S1F) or of NF-κB-dependent antimicrobial peptide genes by *Listeria monocytogenes* (fig. S2) was not affected in the RXN mutant. We noted that *dSTING* expression was still induced by DCV infection in RXN mutant flies, reaching levels close to uninfected wild type three days post infection (dpi, fig. S1B). We hypothesize that a residual level of dSTING protein in the mutant accounts for some remaining activity of the pathway since neither the promoter nor the open reading frame (ORF) of the short form of dSTING are affected by the RXN deletion (fig. S1A).

We next analyzed whether the dSTING pathway could be activated by naturally occurring CDNs known to be agonists of STING in other organisms. Injection of c-di-AMP, 3'3'-cGAMP and 2'3'-cGAMP into WT flies led to a dose-dependent increased expression of *dSTING* and *srg1-3* at 6 and 24 hours post injection (hpi) (Fig. 1A-H and fig. S3). Only c-di-GMP did not trigger a response in these experiments (Fig. 1 and fig. S3, S4). These effects were recapitulated in a cellular model (fig. S5). The induction of *srg1-3* by CDNs was reduced in RXN mutant flies at 6 hpi or abolished at 24 hpi (Fig.

1B-D and F-H). For *dSTING* itself, the pattern of induction was similar in RXN and WT flies, although the level of expression was always substancially reduced in mutant flies (Fig. 1A,E). Induction of *dSTING* and *srg1* was completely abolished in *dSTING* null mutant flies independently generated using CRISPR (*dSTING*^{L76GfsTer11}) (fig. S6A,B). Finally, induction of *dSTING* and *srg1-3* after 2'3'-cGAMP injection was restored in *dSTING*^{Rescue} flies (fig. S6C-F).

Induction of *srg1* and *srg2* by 2'3'-cGAMP was rapid, peaking at 3 or 6 hpi and decreasing afterwards (Fig. 1I,J). Interestingly, inducible expression of *srg3* remained high at 24 hpi (Fig. 1K). Induction of *dSTING* and *srg1-3* by 2'3'-cGAMP was reduced or abolished in *Relish* null mutant flies (Fig. 1L-O), even though the basal level of *dSTING* was not altered (Fig. 1L). Overall, these data reveal that a subset of naturally occurring CDNs can trigger gene expression in *Drosophila*, in a manner dependent on dSTING and Relish.

2'3'-cGAMP has a significant impact on the transcriptome of whole flies

Next, we performed genome-wide transcriptomic analysis to identify 2'3'-cGAMP regulated genes in whole flies. We identified 427 stimulated and 545 repressed genes, displaying at least 1.5-fold change in animals injected with 2'3'-cGAMP compared to Tris buffer (Fig. 2A), with 269, 88 and 115 transcripts stimulated and 311, 53 and 63 transcripts repressed at the 6, 12 and 24h timepoints, respectively (fig. S7). In contrast, only four stimulated and one repressed transcripts were observed when c-di-GMP was injected into WT flies (Data File S1). Clustering analysis revealed three broad categories of stimulated and repressed genes based on their early, sustained or late kinetics of induction or repression (Fig.2B, Data File S2). Among stimulated genes,

srg1 was induced rapidly, while *srg3* classifies as a late induced gene, confirming our initial observation. Rapidly induced genes included antimicrobial peptides, cytokines such as *spaetzle* and *upd3*, transcription factors (e.g. *Rel*, *kay*, *Ets21C, FoxK*) and other signaling molecules (*Takl1*, *pirk*, *Charon*, *dSTING*) (Fig.2C). One of the three canonical components of the siRNA pathway, *AGO2*, was rapidly induced by 2'3'-cGAMP, together with *pst* and *ref(2)P*, which encode restriction factors against picorna-like viruses[28,29] and rhabdoviruses[30], respectively. Late induced genes were mainly unknown but included the JAK-STAT regulated gene *vir-1* and the antiviral gene *Nazo* (Fig. 2C)[5,31]. Gene ontology analysis revealed that the early and sustained stimulated genes were significantly enriched for genes involved in immunity (Fig. 2D). No such enrichment was detected in the late induced genes. By contrast, the 2'3'-cGAMP repressed genes were associated with mitochondria or belonged to several metabolic pathways, including carbohydrate, lipid and protein metabolism (Fig. 2D). This points to an impact of CDN injection on metabolism, possibly reflecting cellular reprograming.

Then, we performed in silico analysis of predicted binding sites for transcription factors in the stimulated genes. We found that 75% of the stimulated genes (321) contained binding sites for members of the NF-κB family. While 84% of early and 80% of the sustained genes contained NF-κB binding sites, only 57% (63) of the late genes contained such binding sites, suggesting a distinct secondary response at 24h post-cGAMP injection. We further analyzed a subset of genes and confirmed that they were induced by 2'3'-cGAMP but not c-di-GMP, and that this induction was dependent on the NF-κB transcription factor Relish (fig. S8).

We could find enrichment for binding sites for other transcription factors that were stimulated by 2'3'-cGAMP, or for transcription factors regulated by induced cytokines (e.g. upd3) (Fig. 2E). Among these, STAT appears to play an important role in all temporal expression profiles, with binding sites in 22%, 42% and 8% of the genes in the early, sustained and late categories, respectively. Others, such as Ets21c, E2F1 and AP1 may participate in the early phase of the response to 2'3'-cGAMP, given their enrichment only in the early and sustained stimulated genes (Fig. 2E).

Injection of 2'3'-cGAMP protects flies against viral infections

We next addressed the functional consequences of activation of the dSTING pathway by CDN injection. Co-injection of 2'3'-cGAMP with DCV or the related cricket paralysis virus (CrPV) resulted in a significant decrease of viral RNA accumulation in WT flies (Fig. 3A-B). Such a protective effect of 2'3'-cGAMP was not observed in RXN mutant flies, but was restored in *dSTING*^{Rescue} flies (fig. S9), indicating that it was dependent on dSTING. Accordingly, 2'3'-cGAMP improved the survival of DCV infected WT flies but not of RXN mutants (Fig. 3C). Co-injection of 2'3'-cGAMP but not c-di-GMP also resulted in reduced accumulation of viral RNA for three other viruses, namely the positive strand RNA virus Flock house virus (FHV), the negative strand RNA virus vesicular stomatitis virus (VSV), and the double strand DNA virus Kallithea virus (KV) (Fig. 3D-F). Collectively, these results indicate that 2'3'-cGAMP triggers protection against a broad range of viruses.

2'3'-cGAMP acts independently of the siRNA response and autophagy, but depends upon the NF-κB transcription factor Relish for its antiviral role

184 To identify the mechanism by which 2'3'-cGAMP exerts its antiviral activity, we first
185 analyzed the effect of CDNs on DCV and VSV infection in *AGO2* null mutant flies. We
186 observed a reduced accumulation of viral RNAs when 2'3'-cGAMP was co-injected
187 with the viruses in both mutant and control flies, revealing that the antiviral function of
188 the CDN does not depend on this key component of the antiviral siRNA pathway (Fig.
189 4A,B). Similarly, 2'3'-cGAMP substancially reduced viral RNA accumulation in *Atg7*
190 null mutant flies, ruling out an involvement of the canonical autophagy pathway (Fig.
191 4C). By contrast, the protective effect of 2'3'-cGAMP against DCV, CrPV and VSV was
192 completely abolished in *Relish* mutant flies (Fig. 4D-F). Altogether, these results reveal
193 that 2'3'-cGAMP triggers a dSTING-NF-κB-dependent antiviral transcriptional
194 response, independent from RNA interference or autophagy.
195

DISCUSSION

CDNs activate antiviral immunity in Drosophila

Our results reveal that three out of the four naturally occurring CDNs that activate mammalian STING can also trigger the dSTING signaling pathway in flies. They raise the question of the mechanism by which 2'3'-cGAMP activates dSTING. Like others[26], we have not been able to detect binding of 2'3'-cGAMP to purified recombinant dSTING. Native purification of the dSTING ligand binding domain expressed in *E. coli* or denaturative purification from *E. coli* inclusion bodies followed by in vitro refolding, resulted in aggregation-prone unstable proteins. This suggested us that the purified protein was not folding correctly. By contrast, we had no difficulties in purifying various mammalian versions of STING using published protocols. The recently reported cryoEM structure of full length chicken STING reveals substantial interaction of the ligand binding domain with areas of the transmembrane domains at the N-terminus of the protein[32]. We believe that such interaction may be critical for either ligand binding or stability (or both) of the cytosolic domain of dSTING, a hypothesis supported by the sequence divergence between the transmembrane domains of STING in mammals and drosophila.

Our work complements the molecular study of Kranzusch and colleagues, who reported binding of CDNs to STING from the sea anemone *N. vectensis*, and supports the hypothesis that the ancestral function of STING in metazoans was to sense CDNs[26]. Bacteria produce a diversity of CDNs and cyclic trinucleotides, some of which could activate dSTING[23,33].Martin *et al* reported that c-di-GMP was able to activate a dSTING-dependent response to *Listeria monocytogenes* infection[23]. However, we did not observe an effect of c-di-GMP upon injection into flies or a contribution of dSTING

to induction of antimicrobial peptides following infection by *L. monocytogenes*. Further experiments comparing mutant alleles and taking other parameters (e.g. microbiota) into consideration are required to clarify the differences between the two studies.

Of note, bacterial CDNs have two canonical 3',5' phosphodiester-linkages, whereas mammalian and *Nematostella* cGAS produce chemically distinct CDNs containing one 2',5' phosphodiester bond joining G to A and one canonical 3',5'- phosphodiester bond joining A to G[21,26]. While we detected activity of 3'3'-CDNs, namely of 3'3'-cGAMP and of c-di-AMP, the strongest agonist was 2',3'-cGAMP, suggesting that an enzyme producing this CDN exists in insects. Indeed, Wang and colleagues recently reported the inducible production of cGAMP in the cytosol of *Bombyx mori* cells infected with nucleopolyhedrovirus (NPV)[24]. Thus, the production of CDNs in the response to virus infection appears to be ancient, possibly inherited in early eukaryotes from prokaryotes[22,34]. A major goal for future study will be the identification and characterization of the cGAS enzyme operating in *Drosophila*.

Activation of NF-κB is an ancestral function of the dSTING pathway

One major difference between STING in mammals and invertebrates, e.g. *Nematostella* and *Drosophila*, is the lack of the CTT domain that mediates interaction with and activation of the kinase TBK1 and the IRF3 transcription factor[22]. This has led to the hypothesis that invertebrate STING regulates autophagy rather than a transcriptional response. Indeed, STING activates autophagy through a mechanism independent of TBK1 activation and IFN induction in mammals. Furthermore, NvSTING also induces autophagy when it is ectopically expressed in human cells[21]. In *Drosophila*, autophagy was found to participate in the control of some viruses, but not

244 others and the effect was modest compared to RNA interference[35,36]. Recently,
245 dSTING-dependent autophagy has been proposed to restrict Zika virus infection in the
246 brain, although autophagy constituents are proviral for Zika and other flaviviruses in
247 mammalian cells[25,37]. Our results using *ATG7* mutant flies indicate that 2'3'-cGAMP
248 can control viral infection independently from the canonical autophagy pathway, but
249 requires both dSTING and Relish. However, we cannot rule out a virus-specific (e.g.
250 Zika virus) role and the involvement of an unconventional autophagy pathway. Indeed,
251 LC3 lipidation in response to cGAMP stimulation in human cells does not depend on
252 the ULK kinases or Beclin 1, two essential components of the classical autophagy
253 pathway[21]. In this regard, we note that one of the genes stimulated by cGAMP is *ref(2)P*,
254 the ortholog of the autophagy receptor p62 and a restriction factor for Sigma virus[30].
255 Even though we cannot completely rule out a contribution of autophagy, our results
256 point to the central role played by the NF-κB transcription factor Relish in the antiviral
257 response triggered by 2'3'-cGAMP. Further analysis will be required to precisely define
258 the contribution of Relish in this response. The dSTING-dependent transcriptional
259 response to cGAMP injection is complex, involving stimulation and repression of gene
260 expression occurring in different waves, with early and late responses. However, the
261 presence of consensus binding sites for NF-κB in the *cis*-regulatory regions of ~75%
262 of the stimulated genes, regardless of their kinetics of induction, confirms a major
263 contribution of Relish. In addition, we identified 13 other transcription factors and 2
264 cytokines (*upd3* and *spz*) in the early and sustained stimulated genes (Data File S3).
265 Among the stimulated transcription factors, *kay* (the Drosophila ortholog of c-Fos),
266 *Ets21C* and *FoxK* were previously implicated in immune, inflammatory or stress
267 responses in Drosophila[38-40]. The *cis*-regulatory regions of the differentially expressed

genes were enriched for binding sites for the mentioned transcription factors and STAT92E, the sole Drosophila STAT ortholog (Fig. 3D, Data File S4). These different transcriptional regulators may coordinate the kinetics of the response and induction of different sets of genes in the context of bacteria and virus infection.

A broad antiviral induced response in *Drosophila*

We observed a striking antiviral activity of 2'3'-cGAMP against a broad range of viruses with DNA or RNA genomes. This contrasts with previous studies that reported virus-specific induced responses[2,41-45], leading to the idea that RNA interference is the only pathway acting on the broad range of viruses infecting invertebrates, which are devoid of interferons. In this regard, we showed that the antiviral effect of 2'3'-cGAMP does not require AGO2, a key component of the antiviral RNAi pathway in flies, even though this gene is stimulated by the CDN. Thus, besides RNAi, an induced antiviral response involving dSTING contributes to host defense against a range of viruses in *Drosophila*. Furthermore, the induction of AGO2 by CDNs suggest a crosstalk between the two pathways where activation of dSTING may potentiate the siRNA response. Intriguingly, while our data are consistent with 2'3'cGAMP triggering dSTING-dependent antiviral immunity, it is less clear that viral infection in flies is capable of inducing CDN production and STING-dependent responses. In particular, we did not observe increased DCV replication in *dSTING* and *Relish* mutant flies, in contrast to what we previously reported[5]. The reason for this discrepancy is not clear at present, but may involve changes in the microbiota of the flies. Indeed, we note that several of the dSTING- and IKKβ-dependent genes that we identified can be regulated by the microbiota[46]. Our previous results pointed to a specific contribution of the dSTING-

IKKβ-Relish pathway in resistance to DCV and CrPV, although a significant but smaller effect was visible also for VSV[5]. This apparent discrepancy could be explained by differences between viruses in the induction of the pathway based on their tissue tropisms, the type of virus-associated molecular pattern produced or the existence of escape strategies, all of which may be bypassed by systemic injection of 2'3'-cGAMP. A number of previous studies reported strong transcriptional responses to virus infection in insects[2,27,31,43,47,48], but also *C. elegans*[49], oysters[50] and shrimps[51]. Analysis of the transcriptional response to viral infections in vivo is complicated by the fact that (i) cell infections are unsynchronized; (ii) host cells are modified through hijacking of cellular functions by viruses; and (iii) many viruses trigger cell lysis and tissue damage, making it complicated to discern the immune response from the non-specific response to stress. Consequently, the transcriptome of virus-infected flies only provides a blurred image of the induced antiviral response[2,27,31,43,44,48]. Identification of an agonist of dSTING bypasses the need for the use of viruses and provides a much clearer picture of the modifications of the drosophila transcriptome associated with induction of antiviral immunity. In particular, our data suggest that 2'3'-cGAMP triggers the expression of cytokines (e.g. Spaetzle, upd3) that amplify the response and trigger expression of antiviral effectors (e.g. Nazo, vir-1). The tools are now at hands to characterize the induced mechanisms controlling viruses in insects, which may reveal original targets for antiviral therapy.

Materials and Methods

Drosophila strains

315 Fly stocks were raised on standard cornmeal agar medium at 25°C. All fly lines used
316 in this study were *Wolbachia* free. w^{1118} , $dSTING^{Control}$, $dSTING^{Rxn}$, *yellow (y) white*
317 *(w) DD1, yw;AGO2*^{414}*,* $Atg7^{d14}$*/Cyo-GFP*, $Atg7^{d77}$*/Cyo-GFP* and $CG5335^{d30}$*/Cyo-GFP*
318 stocks have been described previously[52]*.* $Relish^{E20}$ flies isogenized to the DrosDel
319 w^{1118} isogenic background were a gift from Dr. Luis Teixeira (Instituto Gulbenkian de
320 Ciência)[45].
321 $dSTING^{L76GfsTer11}$ mutants were generated by CRISPR/Cas mediated mutagenesis in
322 a *yw* mutant background. The four base-pairs deletion was verified by Sanger
323 sequencing (Eurofins Genomics), using the sequencing primers described in table S5.
324 Crossing schemes and detailed injection protocols are available upon request.
325 The genomic rescue of wild-type dSTING was established by PhiC31 mediated
326 transgenesis. The fosmid FlyFos015653[53] was injected into the y^1 w^{1118}; *PBac{y[+]-*
327 *attP-9A}VK00027* (BDSC#9744) line and introgressed into a $dSTING^{Rxn}$ mutant
328 background by standard genetic crossing techniques. Transgenesis and initial
329 recombinant fly selection was done by the company BestGene.
330
331 **Virus infection**
332 Viral stocks were prepared in 10 mM Tris-HCl, pH 7.5. Infections were performed with
333 3–5 days old adult flies by intrathoracic injection (Nanoject II apparatus, Drummond
334 Scientific) with 4.6 nL of DCV solution (500 PFU/fly). Injection of the same volume of
335 10 mM Tris-HCl, pH 7.5, was used as a negative control.
336
337 **Bacterial infections**

Listeria monocytogenes (strain 10403S) cultures were grown in brain heart infusion (BHI) medium at 28°C. Infections were performed with 3-5 days old adult flies by intrathoracic injection (Nanoject II apparatus) with 9.2 nL of *L. monocytogenes* solution in PBS (OD600=0.001). The dose used was determined by titration, comparing the wild-type strain to its listeriolysin O-deletion mutant (*L. monocytogenes Δhly*, a kind gift of P. Cossart) to ensure that the response to cytosolic *L. monocytogenes* was monitored[54]. Injection of the same volume of PBS was used as a negative control. Injected flies were kept at 28°C and collected in pools of 6 individuals (3 males + 3 females) at the indicated time points for RNA extraction and RT-qPCR.

CDNs injection with or without viruses

The CDNs (Invivogen) were dissolved in 10 mM Tris pH 7.5 to a concentration of 0.9 mg/mL, and their integrity was monitored by chromatography, as described[55]. 3–5 days old adult flies were CDN stimulated. For CDN injection, each fly was injected with 69 nL of CDN solution or 10 mM Tris pH 7.5 (negative control) by intrathoracic injection using a Nanoject II apparatus. For CDNs and virus coinjection, 30 µL 0.9 mg/mL CDNs were mixed with 2 µL virus (DCV 5PFU/4.6 nL, CRPV 5PFU/4.6 nL, VSV 5000PFU/4.6 nL, FHV 500PFU/4.6 nL and KV). Each fly was injected with 69 nL of CDNs or 10 mM Tris pH 7.5 plus virus mixture by intrathoracic injection using a Nanoject II apparatus (Drummond Scientific) and injected flies were collected in pools of 6 individuals (3 males + 3 females) at indicated time points and homogenized for RNA extraction and RT-qPCR.

CDN transfection of drosophila S2 cells

Drosophila Schneider 2 (S2) cells were seeded in 12 well plates ($2x10^6$ cells per well) in Schneider's Insect Medium (Sigma-Aldrich) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma-Aldrich). 3 h later, the cells were transfected with 10 µg CDN per well using 2 µl Lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturer's protocol. Unsupplemented Schneider's Insect Medium was used for making the transfection complexes. After 6 or 24 hours of transfection, cells were harvested for RNA extraction and qRT-PCR.

RNA extraction and qRT-PCR of *D. melanogaster* tissues

Total RNA from collected flies was extracted using a Trizol Reagent RT bromoanisole solution (MRC), according to the manufacturer's instructions. 1 µg total RNA was reverse transcribed using an iScript™ gDNA clear cDNA synthesis Kit (Biorad), according to the manufacturer's instructions. The DNase and RNA reaction mixture was incubated for 5 min at 25°C to remove genomic DNA and then the reaction was stopped by heating at 75°C for 5min. Then reverse transcription mix was added to DNase-treated RNA template and cDNA was synthesized in the following PCR program: 1) 25°C, 5 min; 2) 46°C, 20 min; 3) 95°C, 1 min. cDNA was used for quantitative real time PCR (qRT-PCR), using iQ™ Custom SYBR Green Supermix Kit (Biorad) according to the manufacturer's instructions and the following qPCR program: 1) 98°C, 15 s; 2) 95°C, 2 s; 3) 60°C, 30 s; 4) plate read; 5) go to step 2, 34X on a CFX384 Touch™ Real-Time PCR platform (Bio-Rad). Primers used for qRT-PCR are listed in table S1. Normalization was performed relative to the housekeeping gene *RpL32*.

386

387 **RNA extraction and qRT-PCR of drosophila S2 cells**

388 Total RNA was extracted using the EZNA Total RNA Kit I (Omega Bio-tek) following
389 the manufacturer's protocol. cDNA was generated with random hexamer primers and
390 the RevertAid RT Reverse Transcription Kit (ThermoFisher Scientific) using 1 µg total
391 RNA as template, following the manufacturer's protocol. cDNA was diluted five times
392 and used as templates for qRT-PCR on a LightCycler® 480 Instrument II (Roche) using
393 LightCycler® 480 SYBR Green I Master reaction mix (Roche) according to the
394 manufacturer's instructions and the following qPCR program: 1) 95°C, 5 min; 2) 95°C,
395 10 s; 3) 55°C; 10 s; 4) 72°C, 10 s; 5) plate read; 6) go to step 2, 44X. Primers used for
396 qRT-PCR are listed in table S1. Normalization was performed relative to the
397 housekeeping gene *RpL32*.

398

399 **RNA-Sequencing of *D. melanogaster* injected with CDNs**

400 Male flies of $dSTING^{Control}$ were injected with 69 nL/fly of either 10 mM Tris (pH 7.5), c-
401 di-GMP (1mg/mL) or 2,3-cGAMP (1 mg/mL) by intrathoracic injection (Nanoject II
402 apparatus), in three independent experiments. Injected flies were collected in pools of
403 6 individuals at 6-, 12- and 24-hours post injection. Total RNA was isolated from
404 injected flies using TRIzol™ Reagent (Invitrogen), according to the manufacturer's
405 protocol. RNA quantity and purity were assessed using a Dw-K5500
406 spectrophotometer (Drawell) and Agilent 2200 TapeStation (Agilent). rRNA was
407 removed using Epicentre Ribo-Zero rRNA Removal Kit (Illumina), and RNA was
408 converted to cDNA. Prepared cDNA was used for Illumina sequencing library
409 preparation using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina

(NEB), following the manufacturer's instructions. Briefly, DNA fragments were end repaired to generate blunt ends with 5′phosphatase and 3′hydroxyls, before adapters ligation, PCR amplification and cleanup. Average fragment length was 300-bp. Purity of the libraries was evaluated using an Agilent 2200 TapeStation. Libraries were used for cluster generation in situ on an HiSeq paired-end flow cell using the Rapid mode cluster generation system, followed by massively parallel sequencing (2×150 bp) on an HiSeq X Ten. Library construction, high throughput sequencing, adapter removal and initial quality control and trimming were done by the company Ribobio.

Transcriptome analysis

After quality trimming and adapter removal using Trimmomatic, reads were mapped using STAR v2.5.3[56] to the Drosophila genome and annotation (ENSEMBL BDGP6.22). Reads mapping to the sense strand of the transcripts were counted with featureCounts v1.6.2[57], using the Drosophila annotation files, allowing mapping to multiple genes. Differential gene expression of transcripts present in ≥20% of the libraries with at least 5 reads across all libraries was done using the *deseq* function of the "DESeq2" (v1.20) package[58]. Variance was estimated using the local fitting method. Read counts and normalized read counts are shown in GEO dataset GSE140955. Transcripts with log2 difference in expression ≥ 1.5 and Benjamini & Hochberg corrected P-value < 0.05 were considered differentially expressed.

Clustering of temporal expression profiles

All differentially expressed genes between Tris and 2'3'-cGAMP injected WT flies at any time point or on average across all time points were clustered in temporal

434 expression categories by partitioning around medoids (PAM) clustering using the *pam*
435 function in the "cluster" (v2.1.0) package. The optimal number of clusters for either
436 stimulated or repressed genes was determined using the gap statistic method, as
437 implemented in the *fviz_nbclust* function of the "factoextra" (v1.0.5) package, using
438 default parameters (100 bootstrapped replications, 10 maximum allowed clusters).
439 Gene expression clusters were visualized using the *Heatmap* function of the
440 "ComplexHeatmap" (v2.0.0) package and *ggplot* of the "ggplot2" (v3.2.1) package.
441
442 **Ontology analysis**
443 Differentially expressed genes between Tris and 2'3'-cGAMP injected WT flies in each
444 temporal expression category were tested for enrichment relative to all genes passing
445 the expression cutoff in any gene ontology type (Molecular Function, Cellular
446 Compartment, Biological Process), using the "Generic GO subset" of gene ontology
447 terms (downloaded from http://current.geneontology.org/ontology/subsets/index.html
448 on 10/10/2019). Gene ontology enrichment analysis was done using the *enricher*
449 function of "clusterProfiler" package (v3.1.12), using default parameters (Benjamini &
450 Hochberg corrected P-value cutoff of 0.05).
451
452 **Transcription Factor Enrichment Analysis**
453 Enrichment of transcription factor binding sites (TFBS) in the regulatory regions of the
454 differentially expressed genes was done using the *cisTarget* function of the "RcisTarget"
455 package (v1.4.0)[59]. The database "dm6-5kb-upstream-full-tx-11species.mc8nr"
456 database was used, which includes the rankings for conserved TFBS in the non-coding
457 regions 5 kb upstream of the transcription start site and in introns of all annotated

458 genes in the D. melanogaster genome (r6.02). Gene symbols were updated to the
459 r6.04 annotation when necessary. Transcription factor family assignment was done
460 according to Flybase (FB2019_05).
461
462 **Statistical analysis**
463 For quantification of viral RNA loads and target gene expression, log transformed ratios
464 were compared using linear regression models using the *lm* function of base R.
465 Survival curves were analysed by Cox regression using the *coxph* function in the
466 "survival" (v2.44-1.1) package. Depending on the experiment, independent variables
467 included genotype, virus injection, CDN injection and time post injection and all
468 interactions between them. Experiment was included as an independent variable in all
469 tests, and the values for each point are shown normalized by adding/subtracting the
470 mean difference between its respective experiment to the grand mean of all log ratios.
471 Multiple comparisons between the groups of interest were done using the *emmeans*
472 function of the "emmeans" (v1.4.1) package, using Dunnett's (for control vs treatment
473 comparisons) or Holm's P value correction. Data were analysed using R (v3.4.2) and
474 *ggplot* was used for plotting.
475
476 **Supplementary Materials**
477 **Figure S1** – DCV infection induces a dSTING dependent transcriptional response in
478 *D. melanogaster.*
479
480 **Figure S2** – Antimicrobial peptide gene induction is not affected in *dSTING* mutant
481 flies after *L. monocytogenes* challenge.

Figure S3 – The cyclic dinucleotides 2'3'-cGAMP, 3'3'-cGAMP and c-di-AMP have a dose dependent effect on the expression of a *dSTING* regulated gene.

Figure S4 – c-di-GMP injection does not induce antimicrobial peptide expression.

Figure S5 – The cyclic dinucleotides 2'3'-cGAMP and 3'3'-cGAMP induce dSTING dependent genes in a cellular model.

Figure S6 – Induction of gene expression following 2'3'-cGAMP injection depends on *dSTING*.

Figure S7 – Differentially expressed transcripts between Tris and 2'3'-cGAMP injected flies in the different timepoints.

Figure S8 – 2'3'-cGAMP induced gene expression is *Relish* dependent.

Figure S9 – A *dSTING* rescue transgene restores 2'3'-cGAMP induced antiviral protection.

Table S1 – List of used olignucleotide primers.

Data S1 – Differentially expressed genes between Tris and c-di-GMP injected $dSTING^{Control}$ flies at 6, 12 and 24 hours post-injection.

Data S2 – Differentially expressed genes between Tris and 2'3'-cGAMP injected *dSTING*^{Control} flies at 6, 12 and 24 hours post-injection.

Data S3 – Differentially expressed transcription factors or cytokines between Tris and 2'3'-cGAMP injected *dSTING*^{Control} flies at 6, 12 and 24 hours post-injection.

Data S4 – Presence of binding sites for stimulated transcription factors in differentially expressed genes.

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718 **FIGURE LEGENDS:**

719

720 **Figure 1**

721 2'3'-cGAMP injection induces a dynamic dSTING-Relish dependent transcriptional
722 response in *D. melanogaster*. Relative gene expression of the indicated dSTING-
723 regulated genes at 6h (**A-D**) and 24h (**E-H**) after injection of Tris and different CDNs in
724 $dSTING^{Control}$ or $dSTING^{Rxn}$ mutant fies. *dSTING* and *srg1-3* were significantly induced
725 in $dSTING^{Control}$ flies 6 hours post injection (hpi) with c-di-AMP, c-di-AMP, 3'3'-cGAMP
726 and 2'3'-cGAMP ($|t| \geq 4.807$, $P < 0.001$ for all comparisons of Tris vs CDN injections).
727 c-di-GMP injection did not lead to significant changes in gene expression at any
728 timepoint ($|t| \leq 0.184$, $P \geq 0.184$ for all comparisons of Tris vs c-di-GMP injected flies).
729 *srg1-3* were never significantly induced in $dSTING^{Rxn}$ mutants 24hpi ($|t| \leq 3.290$, $P \geq$
730 0.200 for all comparisons of Tris vs CDN injections). *dSTING* was induced in
731 $dSTING^{Rxn}$ mutants ($|t| \geq 2.963$, $P \leq 0.017$, for all comparisons of Tris vs CDN injections,
732 excluding c-di-GMP), but the level of expression was always significantly lower than in
733 control flies ($|t| \geq 19.043$, $P < 0.001$ for all pairwise comparisons between control and
734 $dSTING^{Rxn}$). (**I-K**) Expression levels of *srg1-3* at different times post-injection with Tris,
735 cyclic-di-GMP or 2'3'-cGAMP. (**L-O**) Expression levels of *dSTING* and *srg1-3* 6h post-
736 injection with Tris, cyclic-di-GMP or 2'3'-cGAMP in control (w^{1118}) and $w^{1118};Rel^{E20}$ (Rel^{-}
737 $^{/-}$) mutant flies. *dSTING* expression after Tris injection was similar between control and
738 $Rel^{-/-}$ flies ($|t| = 0.659$, $P = 0.515$). After 2'3'-cGAMP injection, induction folds of *dSTING*
739 and *srg1-3* were always significantly lower in Relish mutant than in control flies ($|t| \geq$
740 5.480, $P \leq 0.001$ for all comparison of differences in *dSTING* and *srg1-3* levels between
741 Tris and 2'3'-cGAMP injected flies). Data are from two independent experiments. Each

point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, n.s. – P > 0.05. For panels a-k, comparisons are shown relative to Tris injection in a given genotype or timepoint.

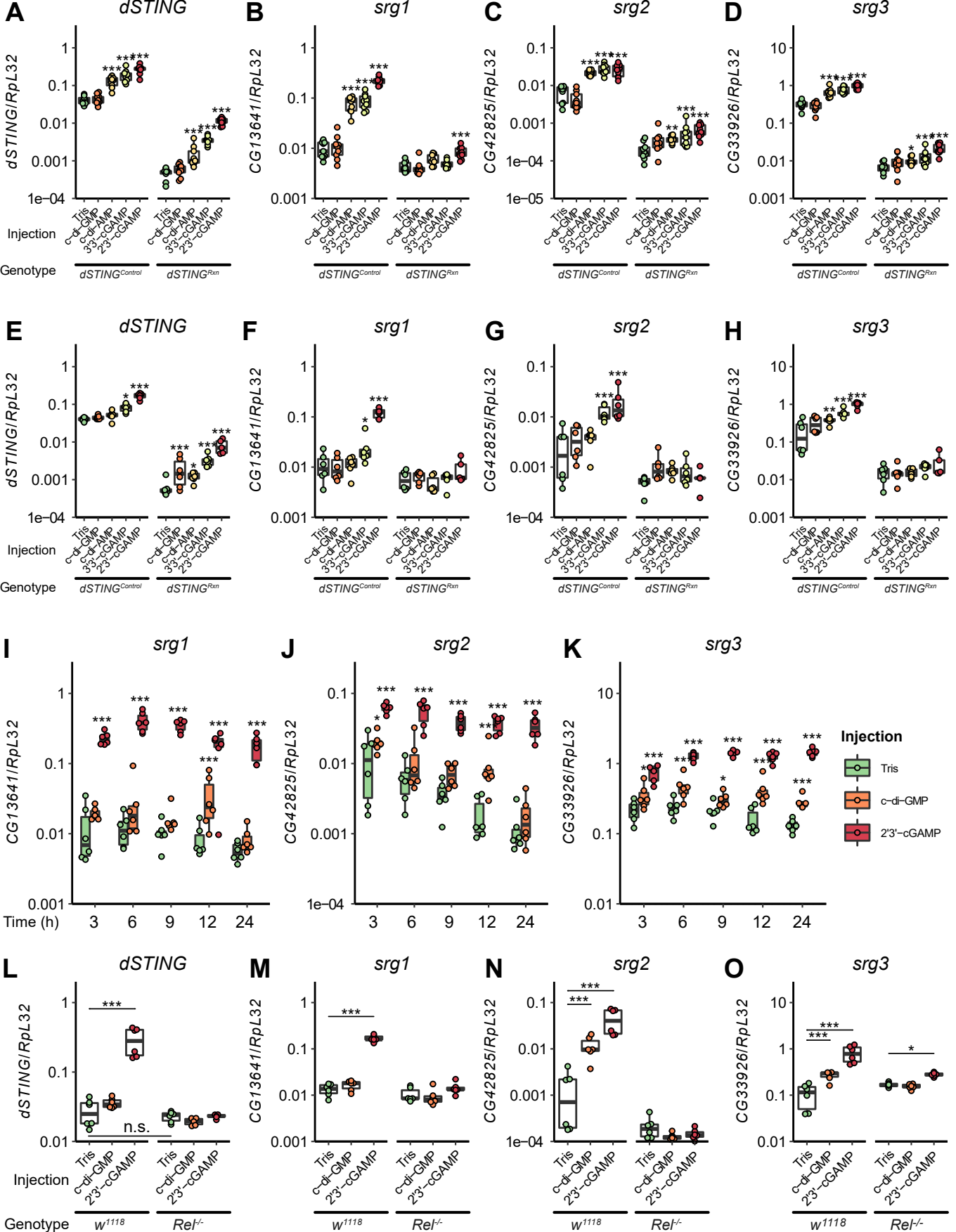
Figure 2

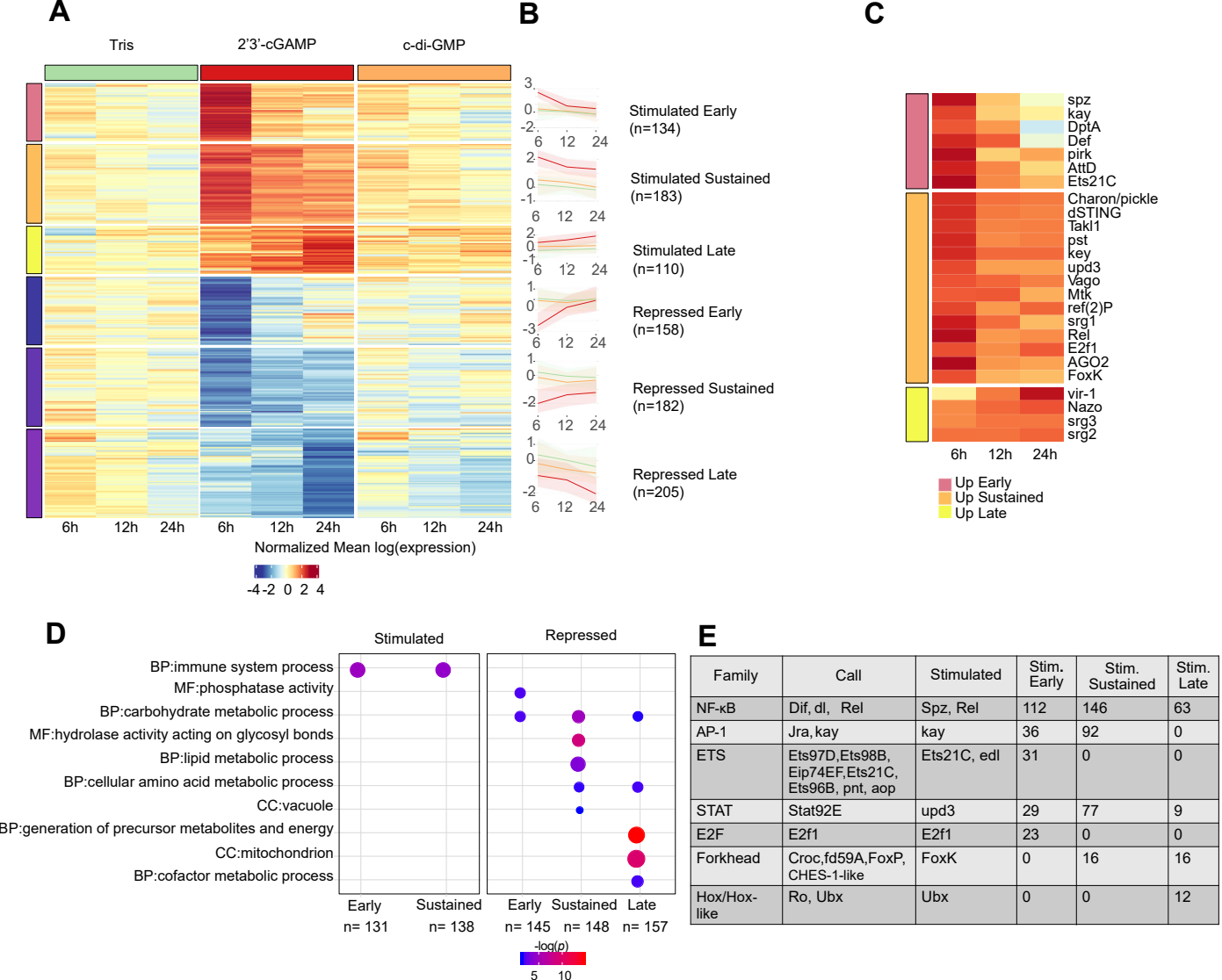
2'3'-cGAMP induces a strong transcriptional response in *D.melanogaster*. (**A**) Expression profiles of $dSTING^{Control}$ flies injected with Tris, 2'3'-cGAMP or c-di-GMP (6, 12 and 24h post-injection). All differentially expressed genes (DEG) between 2',3'-cGAMP- and Tris- injected flies for at least one timepoint or on average across all time points are shown. Values are normalized to the mean log (expression) of Tris-injected flies across the three time points. Expression profiles of stimulated and repressed genes in 2'3'-cGAMP-injected flies were clustered by partition around medoids. (**B**) Normalized mean gene expression by experimental condition in each temporal expression profiles and across the different timepoints. (**C**) Expression of some representative genes discussed in the text. (**D**) Gene ontology enrichment analysis of the DEGs across the different temporal expression profiles. **BP**: Biological process, **MF**: Molecular function, **CC**: Cellular compartment. Size and color of circles indicates respectively the number of DEG and -log(P-value) for the enrichment of each category. (**E**) Numbers of DEGs potentially regulated by **Stimulated** transcription factors and

765 cytokines. Genes with high confidence binding sites for other TFs (**CalI**) of the same
766 family are included.
767
768 **Figure 3**
769 2'3'-cGAMP injection induces a broad, dSTING-dependent, antiviral protection in *D.*
770 *melanogaster*. (**A-C**) Relative DCV (**A**) or CrPV (**B**) RNA loads and survival after
771 infection with DCV (**C**) of $dSTING^{Control}$ and $dSTING^{Rxn}$ mutant flies after co-injection of
772 virus and Tris, 2'3'-cGAMP or c-di-GMP at different days post-injection (d.p.i.). Co-
773 injection with 2'3'-cGAMP resulted in a significant decrease of viral RNA in
774 $dSTING^{Control}$ flies 2 and 3 dpi ($|t| \geq 2.712$, $P \leq 0.020$ for Tris vs 2'3'-cGAMP
775 comparisons and $|t| \leq 0.112$, $P \geq 0.985$ for Tris vs c-di-GMP) but not in mutant flies ($|t|$
776 ≤ 1.547, $P \geq 0.222$) and a significant increase in survival in control but not in mutant
777 flies ($z = 2.404$, $P = 0.032$ and $z = -0.433$, $P = 0.665$ for $dSTING^{Control}$ and $dSTING^{Rxn}$
778 flies, respectively, for the pairwise comparisons between Tris and 2'3'-cGAMP after a
779 Cox proportional hazards model). (**D-F**) Relative viral loads at different time points of
780 control flies after co-injection of Tris, 2'3'-cGAMP or c-di-GMP with the viruses VSV
781 (**D**), FHV (**E**) or KV (**F**). Co-injection with 2'3'-cGAMP, but not c-di-GMP led to a
782 significantly reduced accumulation of all tested viruses ($|t| \geq 2.276$, $P \leq 0.049$ and $|t| \leq$
783 1.769, $P \geq 0.148$ for all pairwise comparisons of Tris vs 2'3'-cGAMP or c-di-GMP at the
784 different days). Data are from two or three independent experiments. For panels a,b
785 and d-f, each point represents a pool of 6 flies. Expression levels are shown relative to
786 the housekeeping gene *RpL32* and are normalized by experiment. Boxplots represent
787 the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points
788 within 1.5 times the interquartile range. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$.

Figure 4

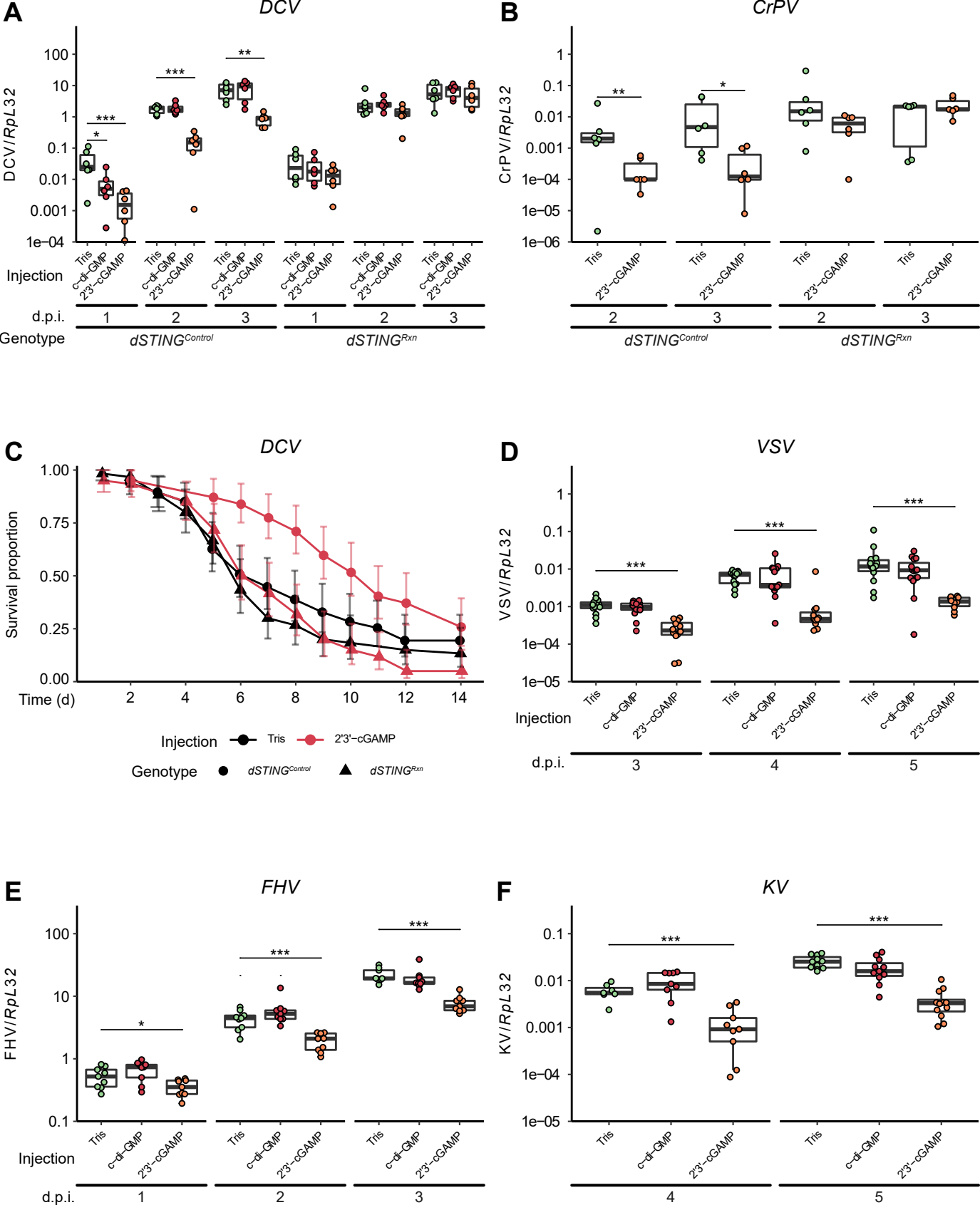
2'3'-cGAMP induced antiviral protection is dependent on Relish, but not on Atg7 or AGO2. Viral RNA loads at different time points after co-injection of Tris or 2'3'-cGAMP with DCV or VSV in flies mutant for the siRNA pathway (*yw;Ago2^{414}* - *Ago2$^{-/-}$*, **A,B**), autophagy (*Atg7$^{d14/d77}$* - *Atg7$^{-/-}$*, **C**), Relish (*w^{1118};Rel^{E20}* - *Rel$^{-/-}$*, **D-F**), or in control flies of the same genetic background (*yw*, *Atg7^{d14}/CG5335^{d30}* - *Atg7$^{-/+}$* or *w^{1118}*, respectively). Co-injection with 2'3'-cGAMP led to a reduced accumulation of viral RNAs in RNAi or autophagy impaired flies (Tris vs 2'3'-cGAMP comparisons, $|t| \geq 2.30$, $P \leq 0.024$ across all timepoints) and in their controls ($|t| \geq 2.53$, $P \leq 0.013$ across all timepoints) but not in Relish mutants ($|t| \leq 1.220$, $P \geq 0.225$ across all timepoints). Data are from two or four (**D**) independent experiments. Each point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment. Triangles indicate points where viral RNA could not be detected: threshold cycles (Cq) values for these points were replaced by the maximum Cq for a virus infected sample + 1. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$.

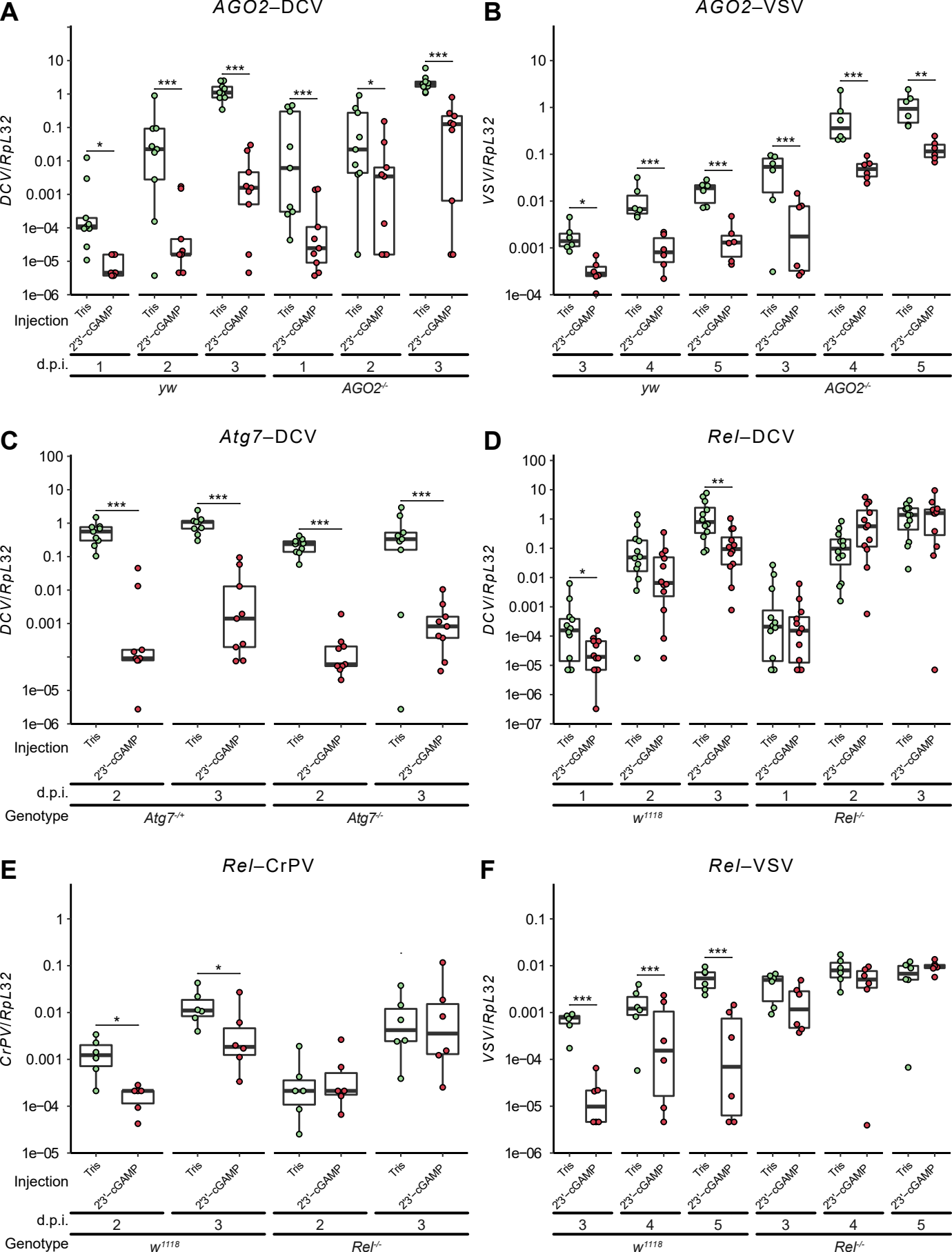

A
dSTING
dSTING/RpL32
B
srg1
CG13641/RpL32
C
srg2
CG42825/RpL32
D
srg3
CG33926/RpL32
Injection
Tris
c−di−GMP
c−di−AMP
3'3'−cGAMP
2'3'−cGAMP
Genotype
dSTING^Control
dSTING^Rxn
E
dSTING
F
srg1
G
srg2
H
srg3
I
srg1
J
srg2
K
srg3
Time (h)
3
6
9
12
24
Injection
Tris
c−di−GMP
2'3'−cGAMP
L
dSTING
M
srg1
N
srg2
O
srg3
n.s.
Genotype
w^1118
Rel^-/-



E

Family	Call	Stimulated	Stim. Early	Stim. Sustained	Stim. Late
NF-κB	Dif, dl, Rel	Spz, Rel	112	146	63
AP-1	Jra, kay	kay	36	92	0
ETS	Ets97D,Ets98B, Eip74EF,Ets21C, Ets96B, pnt, aop	Ets21C, edl	31	0	0
STAT	Stat92E	upd3	29	77	9
E2F	E2f1	E2f1	23	0	0
Forkhead	Croc,fd59A,FoxP, CHES-1-like	FoxK	0	16	16
Hox/Hox-like	Ro, Ubx	Ubx	0	0	12


A
DCV
DCV/RpL32
Injection
Tris
c-di-GMP
2'3'-cGAMP
d.p.i.
Genotype
dSTING^Control
dSTING^Rxn
B
CrPV
CrPV/RpL32
C
DCV
Survival proportion
Time (d)
D
VSV
VSV/RpL32
E
FHV
FHV/RpL32
F
KV
KV/RpL32


A
AGO2–DCV
DCV/RpL32
Injection
Tris
2'3'-cGAMP
d.p.i.
yw
AGO2-/-
B
AGO2–VSV
VSV/RpL32
C
Atg7–DCV
Genotype
Atg7-/+
Atg7-/-
D
Rel–DCV
w1118
Rel-/-
E
Rel–CrPV
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Rel–VSV

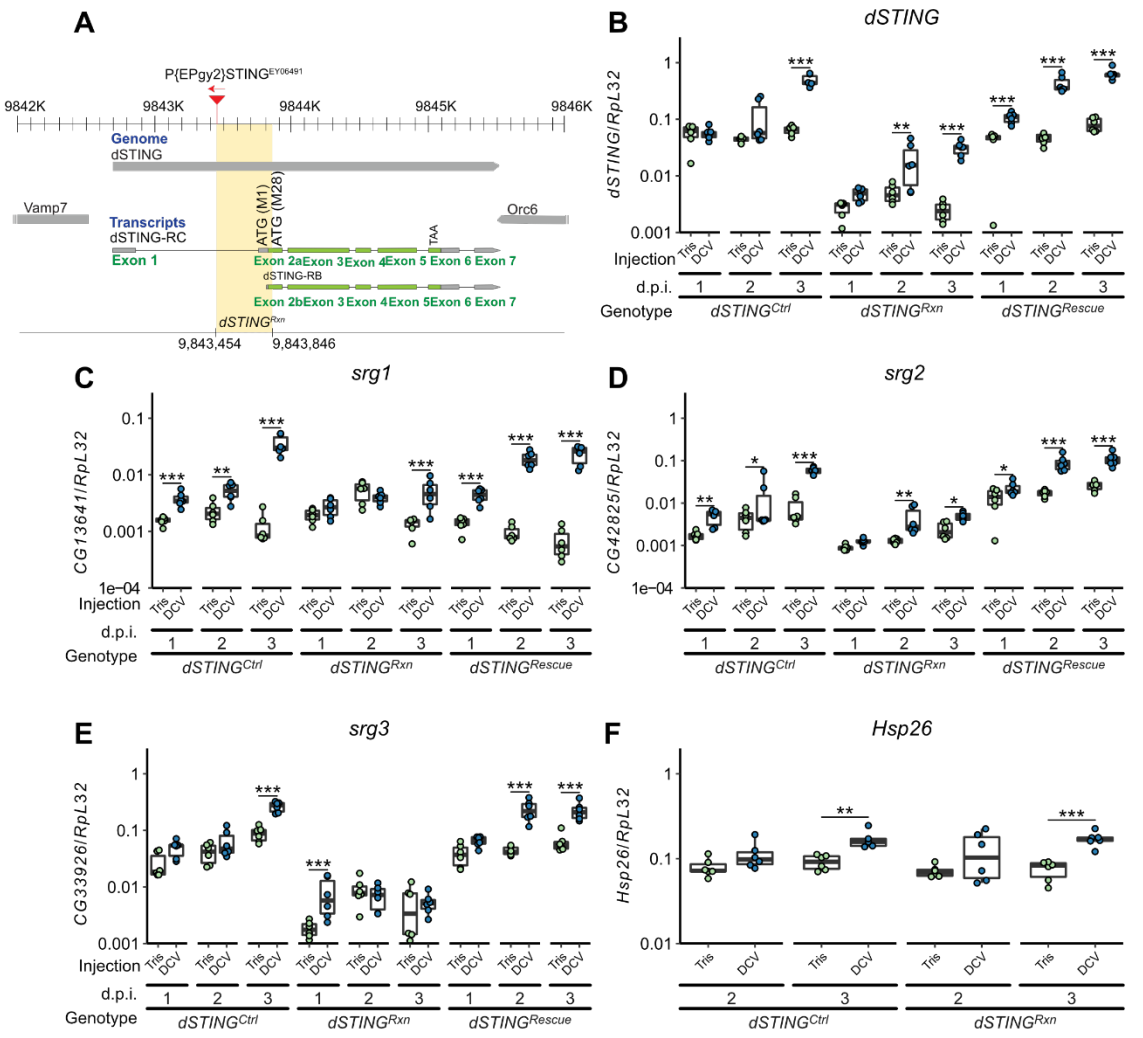

Figure S1 - DCV infection induces a dSTING dependent transcriptional response in *D. melanogaster.*

(**A**) *dSTING*^{Rxn} mutant flies were generated by imprecise excision of the P-element P{EPgy2}Sting^{EY06491}. The boundaries of the deletion (yellow shading), which removes the 3' end of the first intron and the 5' extremity of exons 2a and 2b of the two reported transcripts (RB and RC), are indicated at the bottom. Precise excision of the transposon generated control flies (*dSTING*^{Control}) in the same genetic background. (**B-E**) Relative gene expression at different days post-injection (d.p.i.) of Tris or DCV for *dSTING* (**B**) and *srg1-3* (**C-E**) in *dSTING*^{Control}, *dSTING*^{Rxn} mutant flies and *dSTING*^{Rxn} mutant flies containing a genomic

dSTING rescue transgene (*dSTING*^{Rescue}). Expression of *dSTING* was significanly lower in *dSTING*^{Rxn} mutant ($t \geq -7.189$, $P \leq 0.001$ in all pairwise comparisons between control and *dSTING*^{Rxn} in the different timepoints) and identical to control levels in rescue flies ($|t| \leq 2.044$, $P \geq 0.142$ in all pairwise comparisons between control and *dSTING*^{Rescue} in the different timepoints). STING is induced by DCV infection in *dSTING*^{Rxn} mutant flies ($|t| \geq 3.632$, $P \leq 0.001$ for all pairwise comparisons between Tris and DCV injected *dSTING*^{Rxn}) and reaches levels close to wild type three days post infection ($|t| = 2.466$, $P = 0.065$ for the comparison between DCV injected *dSTING*^{Rxn} and Tris injected control flies). Induction of *srg1* was lower at three dpi in *dSTING*^{Rxn} mutants, stimulation of *srg2* was lower at 3 dpi ($t = 0.6252$, $P = 0.002$) and levels of *srg3* were similar in Tris and DCV infected *dSTING*^{Rxn} mutants 2- and 3- dpi ($|t| \leq 1.268$, $P \geq 0.446$) and always lower than in control flies (> 4.85 fold, $|t| \geq 5.568$, $P < 0.001$). All these genes were induced by DCV infection in control or *dSTING*^{Rescue} flies two or three dpi ($|t| \geq 2.520$, $|P| \leq 0.037$), except for *srg3* in control flies 2 dpi ($t = 1.393$, $P = 0.373$) (**F**) Expression levels of *Hsp26*, a dSTING-independent virus induced gene. Induction of *Hsp26* by DCV was identical in control and *dSTING*^{Rxn} ($|t| \leq 0.842$, $P \geq 0.405$, comparison of differences in *Hsp26* levels between Tris and DCV injected flies at 2 or 3 d.p.i.). Data are from two independent experiments. Each point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$.

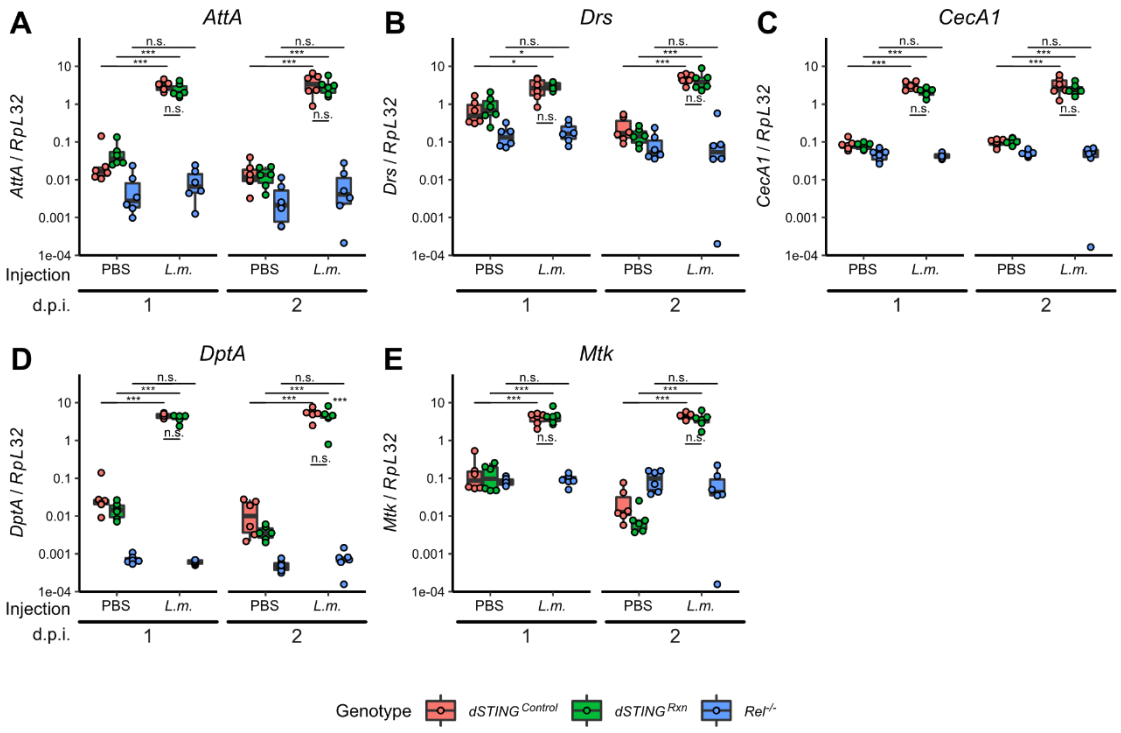

Figure S2 - Antimicrobial peptide gene induction is not affected in *dSTING* mutant flies after *L. monocytogenes* challenge.

(**A-E**) Relative expression of the indicated antimicrobial peptide (AMP) genes one and two days post-injection (d.p.i.) with buffer (PBS) or the gram-negative bacteria *Listeria monocytogenes* (*L.m.*) in control ($dSTING^{Control}$), dSTING ($dSTING^{Rxn}$) or Relish ($Rel^{-/-}$) mutant flies. *L. monocytogenes* infection led to a sustained induction of all tested AMPs in control and dSTING mutant flies but not in Relish mutants (*L. monocytogenes* vs. PBS injection, $|t| \geq 2.503$, $p \leq 0.047$ in control or dSTING mutants and $|t| \leq 2.241$, $P \geq 0.076$ in $Rel^{-/-}$ mutants). Expression levels were similar between control and *dSTING* mutants (control vs. *dSTING* mutants; $|t| \leq 1.911$, $P \geq 0.153$ for all comparisons except for *DptA* 1 d.p.i. after PBS injection $|t| = 2.689$, $P = 0.037$). Data are from two independent experiments. Each point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment.

Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$, n.s. – $P > 0.05$.

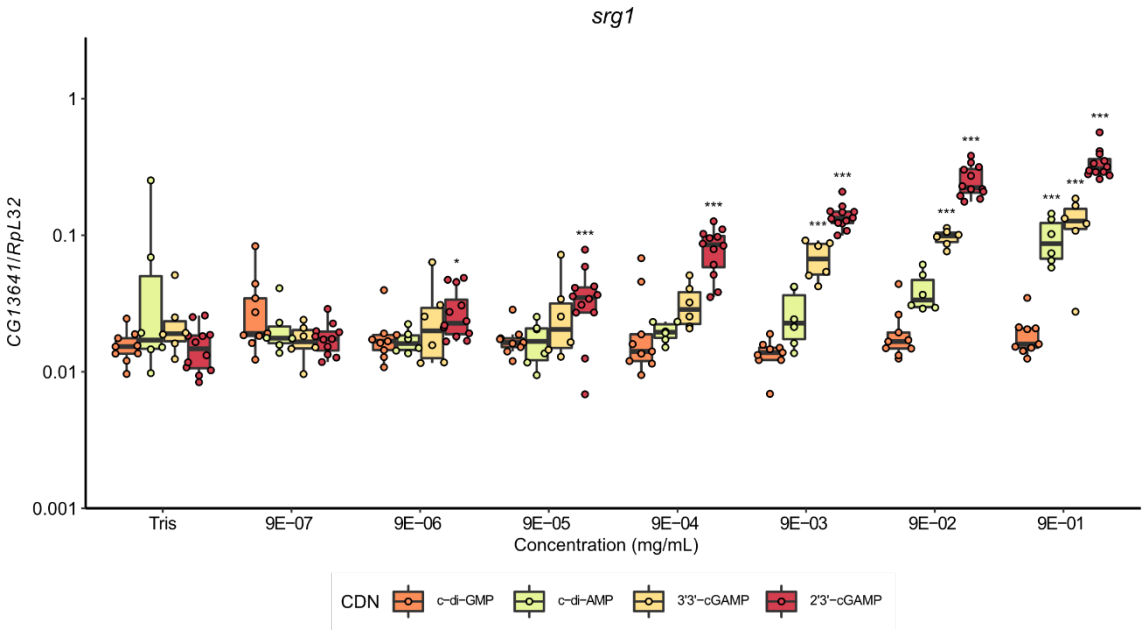

Figure S3 - The cyclic dinucleotides 2'3'-cGAMP, 3'3'-cGAMP and c-di-AMP have a dose dependent effect on the expression of a *dSTING* regulated gene. Relative expression of *srg1* six hours post-injection of buffer (Tris) and the cyclic dinucleotides (CDN) c-di-GMP, c-di-AMP, 3'3'-cGAMP and 2'3'-cGAMP in the indicated concentrations in control flies. *srg1* was induced after injection with concentrations above $9x10^{-6}$ mg/mL of 2'3'-cGAMP (CDN vs matched Tris comparison, $|t| \geq 3.177$, $P \leq 0.011$), $9x10^{-3}$ of 3'3'-cGAMP ($|t| \geq 4.358$, $P < 0.001$) and 0.9 of c-di-AMP ($|t| \geq 4.281$, $P < 0.001$). Injection of c-di-GMP at any concentration did not lead to changes in *srg1* expression ($|t| \leq 2.476$, $P \geq 0.078$). Data are from four (2'3'-cGAMP), three (c-di-GMP) or two independent experiments (3'3'-cGAMP and c-di-AMP). Each point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the

interquartile range. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$. Comparisons are shown relative to the matched Tris injection for a given CDN.

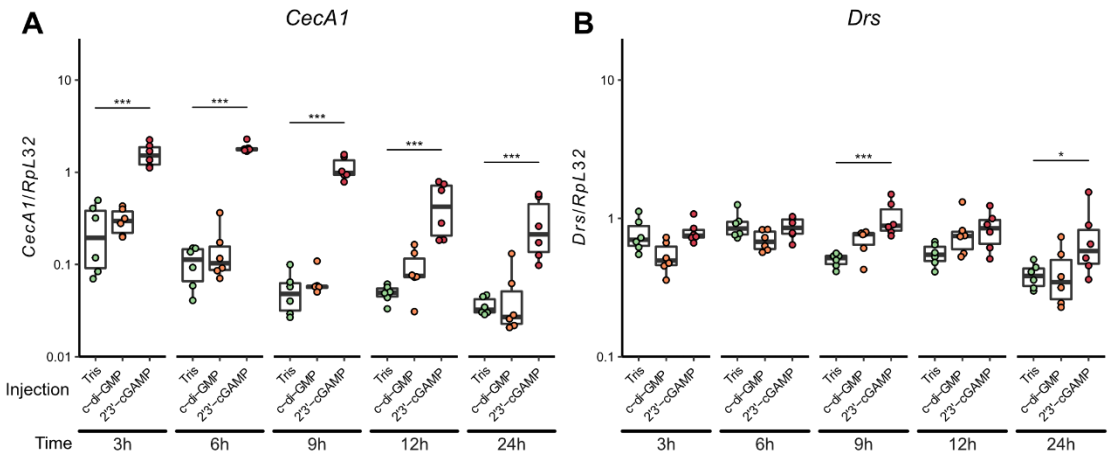

Figure S4 - c-di-GMP injection does not induce antimicrobial peptide expression. (**A-B**) Relative expression of the indicated antimicrobial peptides in control flies across time (h). *CecA1* was sustainedly induced after six or three hours post-injection with 2'3'-cGAMP ($|t| \geq 6.152$, $P < 0.001$) but not after injection with c-di-GMP ($|t| \leq 2.506$, $|P| \geq 0.072$). Data are from two independent experiments. Each point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$.

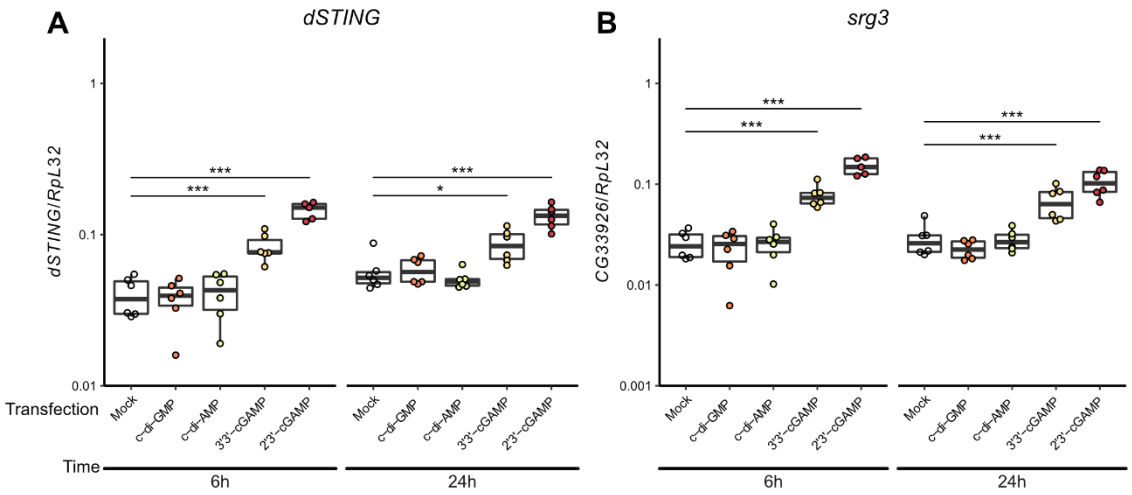

Figure S5 – The cyclic dinucleotides 2'3'-cGAMP and 3'3'-cGAMP induce dSTING dependent genes in a cellular model.

Relative expression of *dSTING* (**A**) and *srg3* (**B**) six and 24 hours post-transfection with Effectene transfection reagent (Mock) and the cyclic dinucleotides (CDN) c-di-GMP, c-di-AMP, 3'3'-cGAMP and 2'3'-cGAMP in drosophila S2 cells. *dSTING* and *srg3* were induced six and 24 hours after transfection with 2'3'-cGAMP and 3'3'-cGAMP (CDN vs Mock $|t| \geq 2.702$, $P < 0.034$). Transfection of c-di-AMP or c-di-GMP at any concentration did not lead to changes in gene expression ($|t| \leq 1.022$, $P \geq 0.673$). Data are from two independent experiments. Each point represents an independent pool of cells. Expression levels are shown relative to the housekeeping gene *RpL32*. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$.

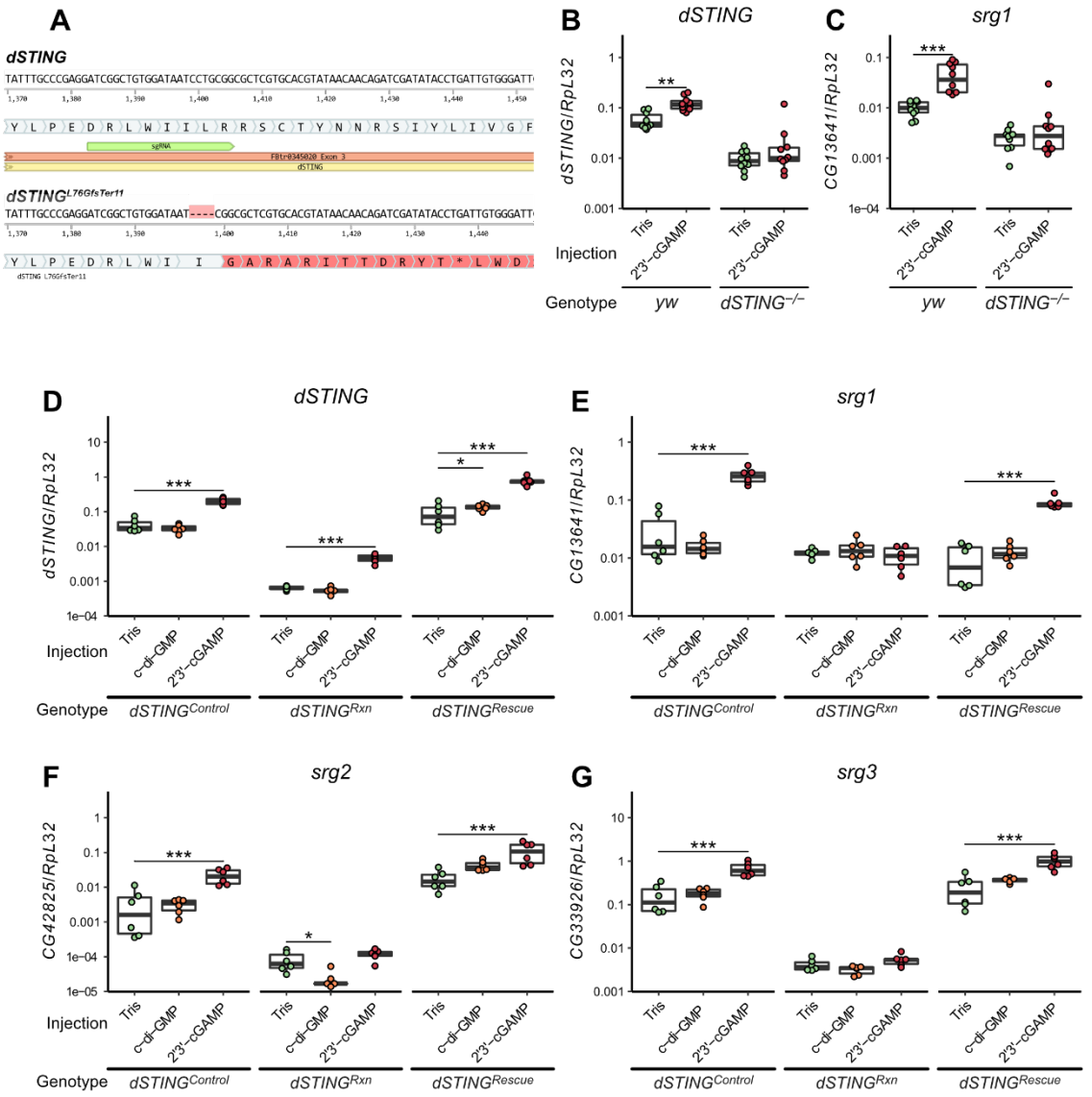

Figure S6 – Induction of gene expression following 2'3'-cGAMP injection depends on dSTING.

(**A**) Sequence of wild-type *dSTING* (top) and *dSTING*^{L76GfsTer11} (bottom) in the vicinity of the sgRNA targeted region (sg). Open reading frame translations are shown below the sequences. Coordinates are in nucleotides, relative to the gene start. (**B,C**) Relative expression of the indicated dSTING-regulated genes at 6h after injection of buffer (Tris) or 2'3'-cGAMP in control (*yw*) or *yw;dSTING*^{L76GfsTer11} (*dSTING*$^{-/-}$) mutant flies. (**D-G**) Relative expression of the indicated dSTING-regulated genes at 6h after injection of buffer (Tris) or 2'3'-cGAMP in control (*dSTING*^{Control}), *dSTING* mutants (*dSTING*^{Rxn}) and *dSTING*

mutants complemented by a genomic rescue of *dSTING* (FlyFos015653;$dSTING^{Rescue}$). *dSTING* and *srg1* were induced by 2'3'cGAMP injection in *yw* flies ($|t| \geq 3.009$, $p \leq 0.01$) but not in $dSTING^{-/-}$ mutants ($|t| \leq 1.561$, $p \geq 0.128$). *srg1-3* were induced after 2'3'cGAMP injection in control and $dSTING^{Rescue}$ flies but not in $dSTING^{Rxn}$ mutant flies (Tris vs. 2'3'cGAMP injections, $|t| \geq 4.359$,$p < 0.001$ in control or $dSTING^{Rescue}$ flies and $|t| \leq 1.102$, $p \geq 0.718$ in $dSTING^{Rxn}$); *dSTING* was induced by 2'3'cGAMP injection in all genotypes ($|t| \geq 7.925$, $p < 0.001$). Data are from three (a-b) or two (c-f) independent experiments. Each point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$.

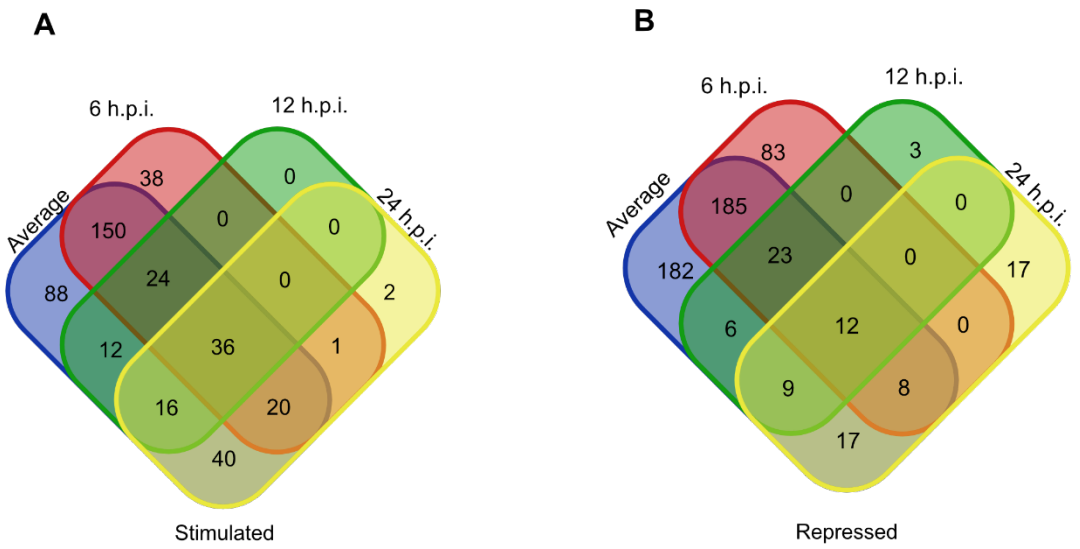

Figure S7 - Differentially expressed transcripts between Tris and 2'3'-cGAMP injected flies in the different timepoints.

Venn diagram of the (**A**) stimulated and (**B**) repressed genes between 2'3'-cGAMP and Tris injected *dSTING*^{Control} flies at the different timepoints (6, 12 and 24h) after injection or on average across all timepoints.

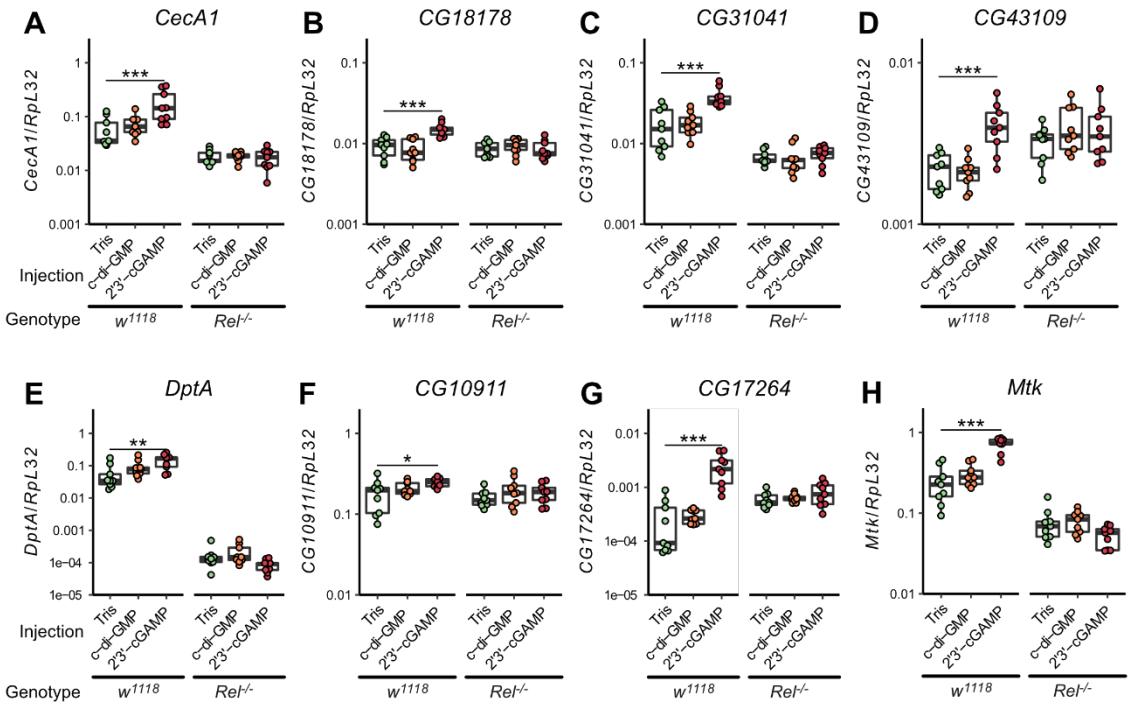

Figure S8 - 2'3'-cGAMP induced gene expression is *Relish* dependent.

Relative expression of the indicated genes six hours post-injection of buffer (Tris), c-di-GMP or 2'3'-cGAMP in control (w^{1118}) or *Relish* ($Rel^{-/-}$) mutant flies. Genes classified as early (**A-E**) or sustained (**F-H**) induced by 2'3'-cGAMP injection according to the RNAseq analysis were induced by 2'3'-cGAMP in control (2'3'-cGAMP vs Tris comparisons, $|t| \geq 2.781$, $P \leq 0.031$) but not in $Rel^{-/-}$ mutants ($|t| \leq 1.932$, $P \geq 0.178$). c-di-GMP injection did not lead to changes in expression of any of the tested genes ($|t| \leq 2.180$, $P \geq 0.102$). Data are from three independent independent experiments. Each point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$.

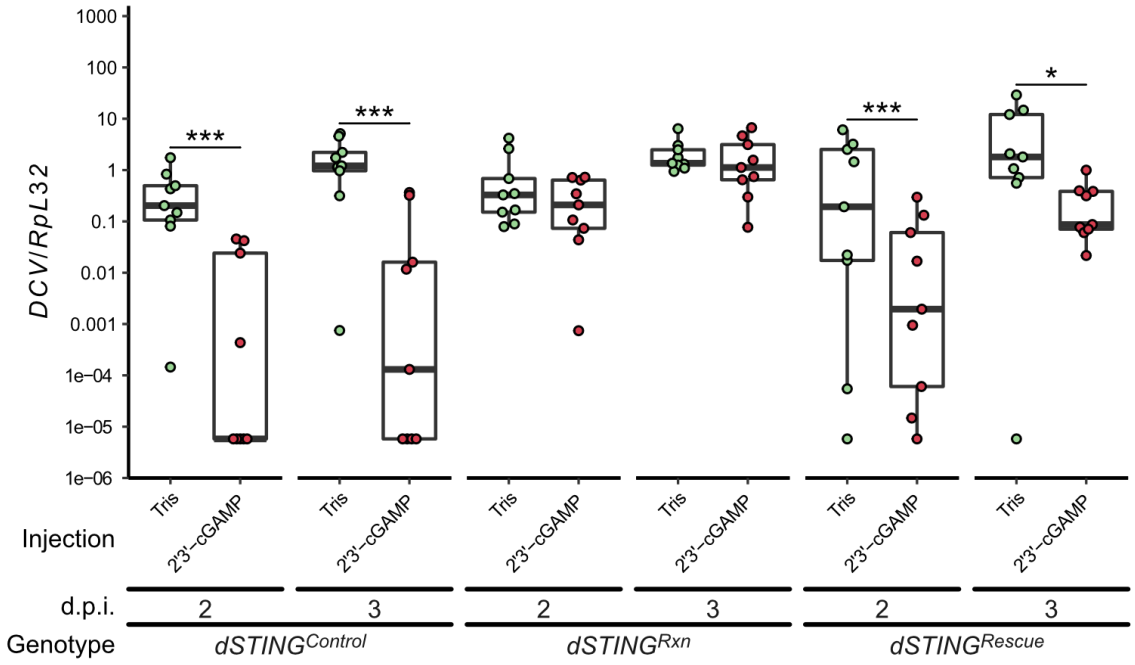

Figure S9 - A *dSTING* rescue transgene restores 2'3'-cGAMP induced antiviral protection.

Relative load of DCV RNA at 2 and 3 days after co-injection (d.p.i.) with buffer (Tris), c-di-GMP or 2'3'-cGAMP in control ($dSTING^{Control}$), *dSTING* mutants ($dSTING^{Rxn}$) and *dSTING* mutants complemented by a genomic rescue of dSTING (FlyFos015653;$dSTING^{Rescue}$). DCV RNA loads were lower after co-injection with 2'3'-cGAMP in control and $dSTING^{Rescue}$ flies but not in $dSTING^{Rxn}$ mutants (Tris vs 2'3'-cGAMP injections, $|t| \geq 2.724$, $P \leq 0.019$ in control or $dSTING^{Rescue}$ flies and $|t| \leq 0.693$, $P \geq 0.976$ in $dSTING^{Rxn}$). Data are from three independent experiments. Each point represents a pool of 6 flies. Expression levels are shown relative to the housekeeping gene *RpL32* and are normalized by experiment. Boxplots represent the median (horizontal line) and 1st/3rd quartiles, with whiskers extending to points within 1.5 times the interquartile range. * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$.

Table S1 - List of used olignucleotide primers.

Target	FlyBase ID	Forward Primer	Reverse Primer	Reference
qRT-PCR				
RpL32	FBgn0002626	GCCGCTTCAAGGGACAGTATCT	AAACGCGGTTCTGCATGAG	[4]
AttA	FBgn0012042	GGCCCATGCCAATTTATTC	AGCAAAGACCTTGGCATCC	[4]
CecA1	FBgn0000276	ACGCGTTGGTCAGCACACT	ACATTGGCGGCTTGTTGAG	[4]
CG13641	FBgn0039239	GTGTCCATTATCCGCACAAG	ACTGGGGTATCTGACGGATG	[4]
dSTING	FBgn0033453	CCGGTGTCTATCGTCCTTTC	CGCTTTAGTTCCTGCATCTG	[4]
CG42825	FBgn0262007	GCGTTTTGGCCCTTATTATG	CTTTTGTAGCCGACGCAGTG	[4]
CG33926	FBgn0053926	GCGACCGTCATTGGATTGG	TGATGGTCCCGTTGATAGCC	[4]
Hsp26	FBgn0001225	CTACAAGGTTCCCGATGGC	GAATACTGACGGTGAGCACG	This work
DCV		TCATCGGTATGCACATTGCT	CGCATAACCATGCTCTTCTG	[4]
CrPV		GCTGAAACGTTCAACGCATA	CCACTTGCTCCATTTGGTTT	[4]
FHV		TTTAGAGCACATGCGTCCAG	CGCTCACTTTCTTCGGGTTA	[4]
KV		CATCAATATCGCGCCATGCC	GACCGAGTTAGCGTCAATGC	[4]
VSV		CATGATCCTGCTCTTCGTCA	TGCAAGCCCGGTATCTTATC	[4]
Drs	FBgn0283461	CGTGAGAACCTTTTCCAATATGATG	TCCCAGGACCACCAGCAT	[60]
DptA	FBgn0004240	GCTGCGCAATCGCTTCTACT	TGGTGGAGTGGGCTTCATG	[60]
Mtk	FBgn0014865	CGTCACCAGGGACCCATTT	CCGGTCTTGGTTGGTTAGGA	[60]
CG18178	FBgn0036035	CGAAGACGAAGATTCCGATGG	TTGGGCTGCGGTTTGATTGTA	This work; https://www.flyrnai.org/flyprimerbank
CG31041	FBgn0051041	ACGTCGAATGCGTGGACTAC	CCGTCGTAATTGTCCTTGCAC	This work; https://www.flyrnai.org/flyprimerbank
CG43109	FBgn0262569	CTCATCCAAGGGCGTTCTGT	TCCCAGGGTGATGATCCCTT	This work; https://www.flyrnai.org/flyprimerbank
CG10911	FBgn0034295	TCCGCCCCTGCAACTTAGTA	TCAAGGGTATGTCCACCATCG	This work; https://www.flyrnai.org/flyprimerbank
CG17264	FBgn0031490	CGTTGCAGGAAATCTCTGATCG	GGGAACAGGGAACAGATGGATAA	This work; https://www.flyrnai.org/flyprimerbank
Sequencing				
dSTING	FBgn0033453	CACCTCTATTCGCATTGTAGC	AGCCGTGAAAGTAGTTGGAG	This work

References - 60

Data S1 - Differentially expressed genes between Tris and c-di-GMP injected *dSTING*^{Control} flies at 6, 12 and 24 hours post-injection.

Columns represent Ensembl gene ID (**gene_id**) and symbol (**gene_symbol**), mean normalized counts after Tris (**TRIS_**) or c-di-GMP injection (**c-di-GMP_**) at the different timepoints (**_06**,**_12** or **_24**), together with estimated $\log_2$(fold-change) (**lfc_**) and Benjamini-Hochberg corrected P-values for the comparison between c-di-GMP and Tris injected flies at each individual timepoint and on average across all timepoints (**_AVG**).

Data S2 - Differentially expressed genes between Tris and 2'3'-cGAMP injected *dSTING*^{Control} flies at 6, 12 and 24 hours post-injection.

Columns represent Ensembl gene ID (**gene_id**) and symbol (**gene_symbol**), temporal expression category (**category**) mean normalized counts after Tris (**TRIS_**) or 2'3'-cGAMP injection (**cGAMP_**) at the different timepoints (**_06**,**_12** or **_24**), together with estimated $\log_2$(fold-change) (**lfc_**) and Benjamini-Hochberg corrected P-values for the comparison between c-di-GMP and Tris injected flies at each individual timepoint and on average across all timepoints (**_AVG**).

Data S3 - Differentially expressed transcription factors or cytokines between Tris and 2'3'-cGAMP injected *dSTING*^{Control} flies at 6, 12 and 24 hours post-injection.

Columns headings are as in data S2, and include the transcription factor family/sub-family (**Family**).

Data S4 - Presence of binding sites for stimulated transcription factors in differentially expressed genes.

Differentially expressed genes between Tris and 2'3'-cGAMP injected *dSTING*^{Control} flies with regulatory sequences enriched for the differentially expressed transcription factors, or for transcription factors of the same family/sub-family. Columns headings are as in data S3, and include the high confidence transcription factor calls predicted by Rcistarget.