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Induced antiviral innate immunity in *Drosophila*

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Abstract

Immunity to viral infections in the model organism *Drosophila melanogaster* involves both RNA interference and additional induced responses. The latter include cellular mechanisms such as programmed cell death and autophagy, but also the induction of a large set of genes, some of which contribute to the control of viral replication and resistance to infection. This induced response to infection is complex and involves both virus-specific and cell-type specific mechanisms. We review here recent developments, from the sensing of viral infection to the induction of signaling pathways and production of antiviral effector molecules. Our current understanding, although still partial, validates the *Drosophila* model of antiviral induced immunity for insect pests and disease vectors, as well as for mammals.

Introduction

Viral infections represent a major burden for all organisms. Not only do they have an important impact on human health, as illustrated by epidemics such as HIV or flu, but they also represent a substantial economic burden, through their effects on crops and animals, including insects such as honeybees. Given that viruses replicate inside cells, the host discrimination between self and non-self presents particular challenges. In addition, the rapid evolution of viruses is manifest in viral mechanisms for suppressing host defenses. Investigating antiviral immunity in a wide range of organisms provides a broad view of the antiviral strategies adopted throughout evolution in different species and can reveal novel therapeutic targets.

An important facet of resistance to viral infections in insects is RNA interference (RNAi), which provides a sequence-specific intrinsic defense against viral infections [1]. In addition, viral infections can trigger cellular responses such as apoptosis or autophagy, and the

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induction of a range of anti-viral gene products. Whereas RNAi is triggered by double stranded (ds) RNA generated during viral replication, little is known about the receptors and mechanisms mediating viral sensing in insects. We therefore start this article by discussing the contribution of inducible responses to the control of viral infection in flies. The contribution of the NF-κB and STAT signaling pathways to antiviral responses and our current understanding of viral sensing in *Drosophila* is reviewed. Potential approaches for further research are identified.

Induced cell death and autophagy contribute to antiviral immunity

Two cellular mechanisms, apoptosis and autophagy, restrict viral replication and dissemination in insects (Fig. 1a, b). Apoptosis is triggered in lepidopteran and *Drosophila* cells in response to infection by the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), and this programmed cell death reduces viral production [2]. Apoptosis is also induced following infection by RNA viruses, such as the Flock House Virus (FHV), a RNA virus belong to the *Nodaviridae* family [3]. Caspases, the proteases that trigger apoptosis, are tightly regulated by the members of the IAP (inhibitor of apoptosis protein) family (e.g. DIAP1 in *Drosophila*) [4]. Four *Drosophila* genes clustered in a small region of the 3rd chromosome (RHG genes: *reaper*, *hid*, *grim* and *sickle*) encode antagonists of IAPs. Expression of these genes is induced in cells destined to die during development, or in response to genotoxic stress. *Drosophila* containing a deletion of the irradiation responsive enhancer region (IRER), which controls expression of the RHG genes, are deficient for apoptosis and are unable to restrain baculovirus or FHV infection in larvae or adults, respectively. Virus-induced apoptosis and control of viral load in infected flies is also impaired in mutants for the transcription factor *p53*, a key regulator of apoptosis [5••].

Autophagy is a conserved process that targets cytoplasmic contents to lysosomes for digestion. This process involves formation of a cup-shaped isolation membrane termed the phagophore, which sequesters a portion of the cytosol. During this process, adaptor proteins like Sequestosome 1 couple the autophagic cargo to the phagophore membrane [6•]. One hallmark of autophagy is the proteolytic processing and recruitment of the protein Atg8/LC3, to dot-like structures in the phagophore. In flies, autophagy participates in the control of two different arthropod-borne viruses (arboviruses), Vesicular Stomatitis virus (VSV) and Rift valley fever virus (RVFV) [7,8]. Interestingly, the gene *ref(2)P*, which encodes the fly homologue of Sequestosome-1, is an important restriction factor for the natural fly pathogen Sigma virus (SIGMAV), a member of the *Rhabdoviridae*, like VSV [9••]. This suggests that *Drosophila* Sequestosome-1 may interact with SIGMAV components, thus triggering autophagy. However, replication of SIGMAV is more efficient in flies homozygous for the sensitive allele of *ref(2)P* than in null mutant flies, indicating that *ref(2)P* can have a proviral, rather than antiviral, role [10].

Virus induced genes and the control of viral infection

Several genome-wide microarray analyses [11–16•] or cells [17–19] indicate that viral infections trigger a transcriptional response. Some overlap exists between the genes induced by viruses and bacteria or fungi. For example, antimicrobial peptides (AMPs) are

upregulated following viral infection [20,21], although not as strongly as in the case of bacterial infections [22].

Understanding the induced response to viral infection is complicated by the poor reproducibility of the transcriptomic data, as shown for two viruses, SINV and FHV. Three independent studies analyzed gene expression in SINV infected wild-type flies [16•], in transgenic flies expressing a SINV-GFP replicon [15], and in infected tissue culture S2 cells [18]. As shown in Fig. 2a, there is little overlap between the induced genes reported in these studies. Besides methodological differences in RNA quantification and data analysis, these discrepancies probably reflect the response of the whole organism *vs* a more homogenous population of tissue culture cells, and differences between *a bona fide* infection and expression of a replicon in transgenic flies, which bypasses essential steps of the viral cycle. It is interesting to note however that four of the five genes induced by both the replicon and SINV infection *in vivo* (*Vago*, *Frost*, *CG11671*, *CG10912*) are induced by other viruses (DCV, FHV, SIGMAV), and that one of them (*Vago*) encodes a protein associated with antiviral activity (see below). In the case of FHV as well, differences between datasets monitoring host gene expression following infection in flies or tissue culture cells are apparent (Fig. 2b).

These limitations notwithstanding, a comparative genome wide microarray analysis revealed that three distinct RNA viruses, DCV, FHV and SINV trigger overlapping but different responses in flies [16•] (Fig. 2c). This study identified 42 genes that are upregulated by all three viruses, but also revealed different patterns of genes induced by DCV on one hand, and FHV and SINV on the other (Fig. 2c, d). These altered expression patterns may reflect either differences in viral replication strategies (e.g. IRES-dependent translation for the picorna-like virus DCV) and tissue tropism, or co-evolution of DCV with its natural host. Interestingly, the existence of virus-specific induced responses is supported by genetic evidence (see below).

Antiviral effectors

One hallmark of the *Drosophila* response to bacterial/fungal infections is the secretion in the hemolymph of a cocktail of AMPs [23]. Two AMP coding genes, *Diptericin* (*Dpt)B* and *Attacin* (*Att)C,* are upregulated in transgenic flies expressing a SINV replicon. Silencing their expression led to a modest but significant increase in SINV replication, suggesting that DptB and AttC have non-redundant antiviral functions [15]. Another *Drosophila* study however reported that the single overexpression of any of the seven canonical AMPs is not sufficient to protect flies against infection by Drosophila X virus (DXV) [20]. In *Aedes aegypti*, a member of the Cecropin family was induced in the salivary glands following Dengue virus (DENV) infection. Biochemical experiments revealed that this Cecropin, in addition to antibacterial activity, also has antiviral activity against both DENV and Chikungunya virus [24].

Besides AMPs, the analysis of the transcriptome of virus-infected insects or cells provides an as yet underexploited list of other antiviral effector candidates. In *Aedes* mosquitoes, microarray analysis led to the identification of two Jak/STAT-regulated genes encoding

proteins with antiviral activity, DENV restriction factors (DVRF) 1 and 2 [25]. Another virus induced factor, the thiol-ester containing protein TEPII, has been proposed to downregulate SINV infection in *A. albopictus* and *Drosophila* cell lines, through its effects on the processing of the non structural polyprotein [18]. Finally, in flies, one gene associated with antiviral activity is *Vago* [26]. Identified among the genes induced by DCV and SINV infection, *Vago* encodes a 18-kilodalton cysteine-rich polypeptide participating in the control of viral infection in the fat body. Interestingly, orthologues of Vago are induced by viral infection in Culex quinquefasciatus and Aedes albopictus cell lines. Furthermore, the *Culex* Vago orthologue opposes replication of the arbovirus West-Nile virus (WNV) in a cell line [27••,28]. In this *ex vivo* cellular model, CxVago, secreted upon WNV infection, appears to activate the Jak/STAT pathway, suggesting that CxVago is an antiviral cytokine.

Virus-specific contribution of NF-κB and STAT signaling pathways in antiviral defense

In *Drosophila*, two pathways, Toll and IMD, regulate transcription factors of the NF-κB family and production of AMPs during bacterial and fungal infections [29]. These pathways may also be involved in antiviral immunity. For example, flies mutant for some genes of the Toll pathway fail to control infection by DENV [30] or DXV [20], although they show normal resistance to SINV infection [21]. The Toll pathway is also involved in the control of DENV infection in the disease-vector mosquito, *Aedes aegypti* [31]. The IMD pathway may participate in the control of two other RNA viruses, Cricket paralysis virus (CrPV) and SINV [21,32].

An unbiased analysis of the promoters of the genes induced by DCV pointed to the involvement of another evolutionarily conserved innate immunity signaling pathway, namely Jak-STAT. Indeed, many DCV-induced genes contain STAT binding sites in their proximal promoters, and flies mutant for the gene *hopscotch*, which encodes the *Drosophila* Jak kinase, are more sensitive to DCV infection than wild-types [13]. Interestingly, resistance of *hopscotch* mutant flies to FHV and SINV, which induce a different transcriptional response than DCV (Fig. 2c) is not affected [16•]. These flies however have increased susceptibility to infection by another virus of the *Dicistroviridae* family, CrPV, indicating that the Jak/STAT pathway is activated upon sensing of a feature specific to picorna-like viruses. Indeed, expression of the cytokines Unpaired (Upd) 2 and 3, which activate the receptor Domeless and the Jak/STAT pathway, is strongly induced following infection by either virus [16•]. The Jak/STAT pathway also participates in the control of viral infection in *Aedes* and *Culex* mosquitoes [25,27••].

Sensing viral molecules by the innate immune system in insects

Innate immunity is activated by “microbial associated molecular patterns”, which are recognized by pattern recognition receptors (PRRs). A molecular pattern typical of viral infections is dsRNA generated during replication. The dsRNA binding protein B2 encoded by FHV prevents induction of the gene *Vago* [26], suggesting that this response to viral infection is triggered by dsRNA. Indeed, induction of *Vago* depends on Dicer-2, which triggers both RNAi and an inducible response upon sensing viral RNAs (Fig. 1c) [26,27••].

Interestingly, the N-terminal DExD/H box helicase domain of Dicer-2 is phylogenetically related to that of the vertebrate RIG-I-like receptors, implying that the involvement of this domain in antiviral innate immunity is an ancient evolutionarily conserved feature [26]. A key issue that is still not resolved is how Dicer-2 triggers gene expression after sensing viral RNAs. In *Culex*, Dicer-2 triggers a pathway including a TRAF protein and the NF-κB protein REL2 [28].

In mammals, some viral proteins are sensed by a different family of PRRs, the transmembrane Toll-like receptors (TLRs) [33]. In *Drosophila*, four Toll receptors function as cytokine receptors [34–36]. In particular, Toll-6, -7 and -8 function as neurotrophin receptors in the central nervous system [35,36]. In addition, Toll-7 has been proposed to recognize the glycoprotein G from VSV, or an as yet unidentified component of the RVFV virion to trigger autophagy (Fig. 1b) [8,37]. It is not yet known if other components of viral particles such as lipids or sugar moieties can be sensed by the innate immune system of the fly.

Sensing stress or altered cell metabolism in virus infected flies

Besides molecular patterns, alteration of cellular functions or danger signals can also activate innate immunity. In the case of baculovirus infection, two mechanisms account for the activation of caspases in infected cells: (i) inhibition of IAP synthesis, resulting from virus-induced shutdown of translation [3,38] and (ii) induction of IAP antagonist proteins (Fig. 1a) [5••]. Baculoviruses and other large DNA viruses trigger a DNA damage response (DDR), possibly as a result of the detection of short-lived stretches of single stranded DNA or dsDNA ends formed in the course of viral DNA replication. This leads to activation of the transcription factor p53 [39,40]. In turn, p53 induces expression of RHG proteins [5••]. The recent discovery that viral DNA forms are generated in FHV infected cells suggests that the DDR may contribute to the response to this RNA virus [41••].

Activation of autophagy by VSV involves the repression of the PI3 kinase/AKT/TOR pathway, which inhibits autophagy under normal conditions [7]. This pathway also plays a role in the control of SINV infection in insect cells [42]. In this case however, SINV activates the pathway, which leads to the phosphorylation of 4E-BP1 and increased translation of viral proteins. Hence, activation of the PI3K/AKT/TOR pathway favors replication of two different viruses in flies, although by distinct mechanisms: repression of autophagy for VSV *vs* activation of cap-dependent translation for SINV. Of note, a major physiological regulator of PI3K/AKT/TOR pathway is insulin, which also activates the ERK pathway. This pathway was recently shown to be activated by viral infection, and to repress replication of DCV, SINV and VSV by an unknown mechanism [43••]. These findings highlight the complex interconnection between pathways regulating cellular metabolism and antiviral immunity in *Drosophila*.

Finally, indirect evidence suggests that tissue damage and release of cell debris can induce an antiviral immune response. Indeed, the DCV and CrPV-induced cytokines Upd2 and -3 [16•], which activate the Jak/STAT pathway, are regulated by stress and tissue damage [44].

The biochemical nature of the stimulus activating expression of Upd cytokines in virus-infected flies remains unknown.

Conclusion and perspectives

Research on antiviral immunity in *Drosophila* over the past ten years has identified a broad range of responses besides RNA interference. In addition, tissue-specific regulation of the cellular environment [45], virus-specific restriction factors [9••,46–48] and endosymbiont bacteria such as *Wolbachia* [30] also have an important impact on the outcome of viral infections in flies. Recent findings indicate that the inducible antiviral responses in *Drosophila* are conserved in mosquito vectors of human disease. In addition, the involvement of an evolutionarily conserved DExD/H box helicase domain in sensing viral RNAs together with the roles of NF-κB and STAT signaling pathways imply that this conservation extends to mammalian systems, thus highlighting the potential of the *Drosophila* viral response model.

Recent progress has provided a number of assays (e.g. processing of caspases or Atg8, induction of gene expression) to monitor activation of antiviral pathways, and characterize the receptors sensing viral infection in flies. Strategies to identify these molecules include a combination of biochemical and genetic approaches, similar to those used for the identification of the PRRs detecting bacterial and fungal infections in insects [29]. For example, purification of proteins binding RNAs with characteristic viral features (e.g. dsRNA, 5’ triphosphate or 2’O-unmethylated extremities), may identify viral sensors, as recently reported in mammals [49]. Gain of function approaches, such as those used recently in mammalian cells to functionally characterize the dozens of interferon stimulated genes (ISGs), may also reveal new receptors (e.g. [50]). Finally, genetics is the greatest asset of *Drosophila*. The major limitation of this approach has been the time consuming positional cloning of mutant genes, but advances in sequencing technology have facilitated the identification of mutations. In consequence, genetic screens to identify mutants impaired in the control of viral infections holds great promise to identify novel host antiviral genes, and, in particular, to identify the receptor molecules that sense viral infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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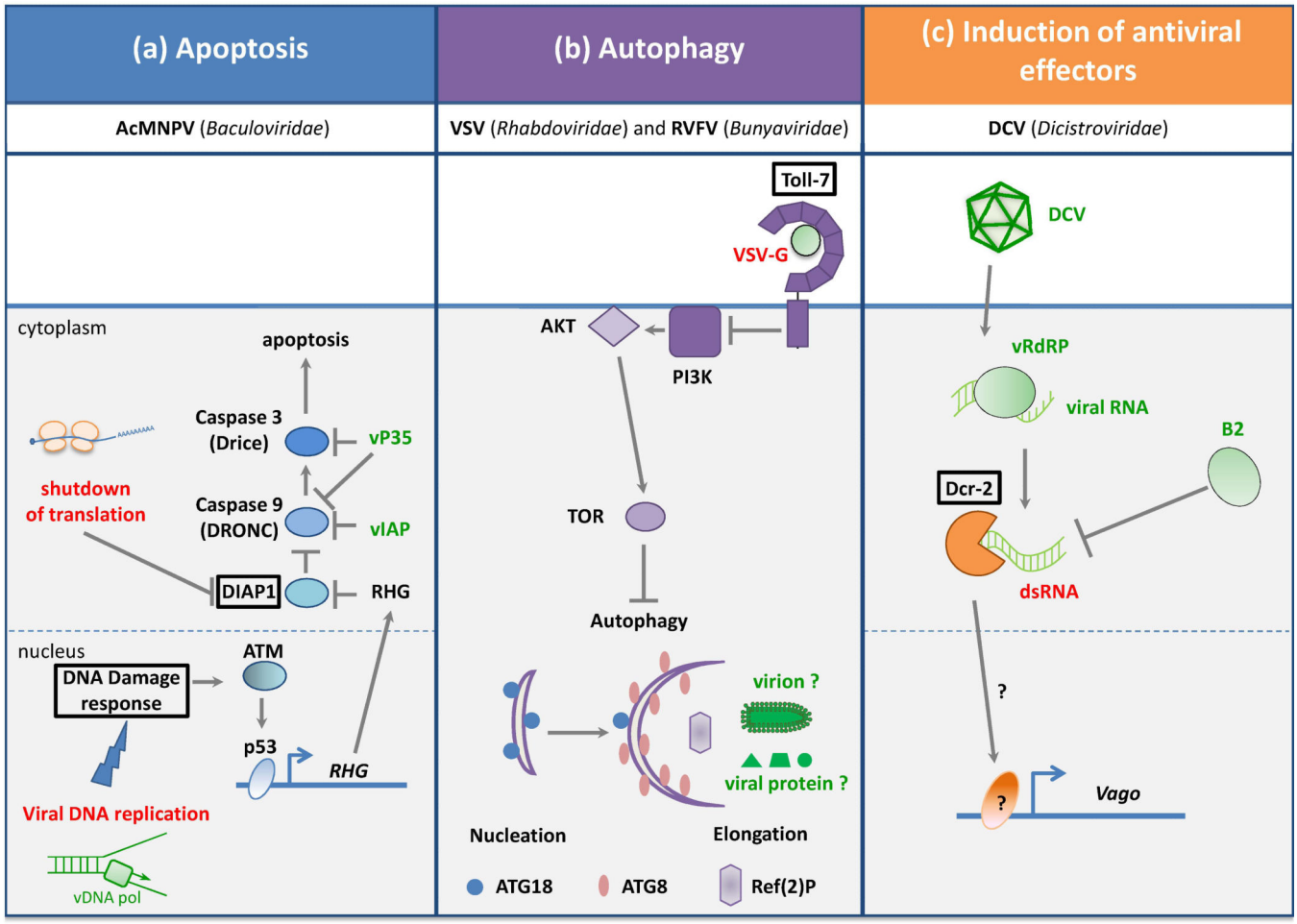

Figure 1. Induced antiviral responses in *Drosophila*

Induction of specific antiviral pathways in *Drosophila melanogaster* triggered by different viruses. (**a**) Apoptosis is induced during the infection by the DNA virus AcMNPV. (**b**) Infection by VSV and RVFV, two negative sense single-stranded (ss) RNA viruses, trigger antiviral autophagy program. The inhibition of PI3K by Toll-7 and the contribution of Ref(2)P remain poorly characterized. (**c**) Induction of antiviral effectors during the infection of the positive ssRNA DCV is mediated by DExD/H box helicase Dicer-2, which senses dsRNA produced by the viral RNA-dependent RNA polymerase (vRdRP). The triggers are indicated in red, the sensors are boxed and the viral components are in green. See the text for details.

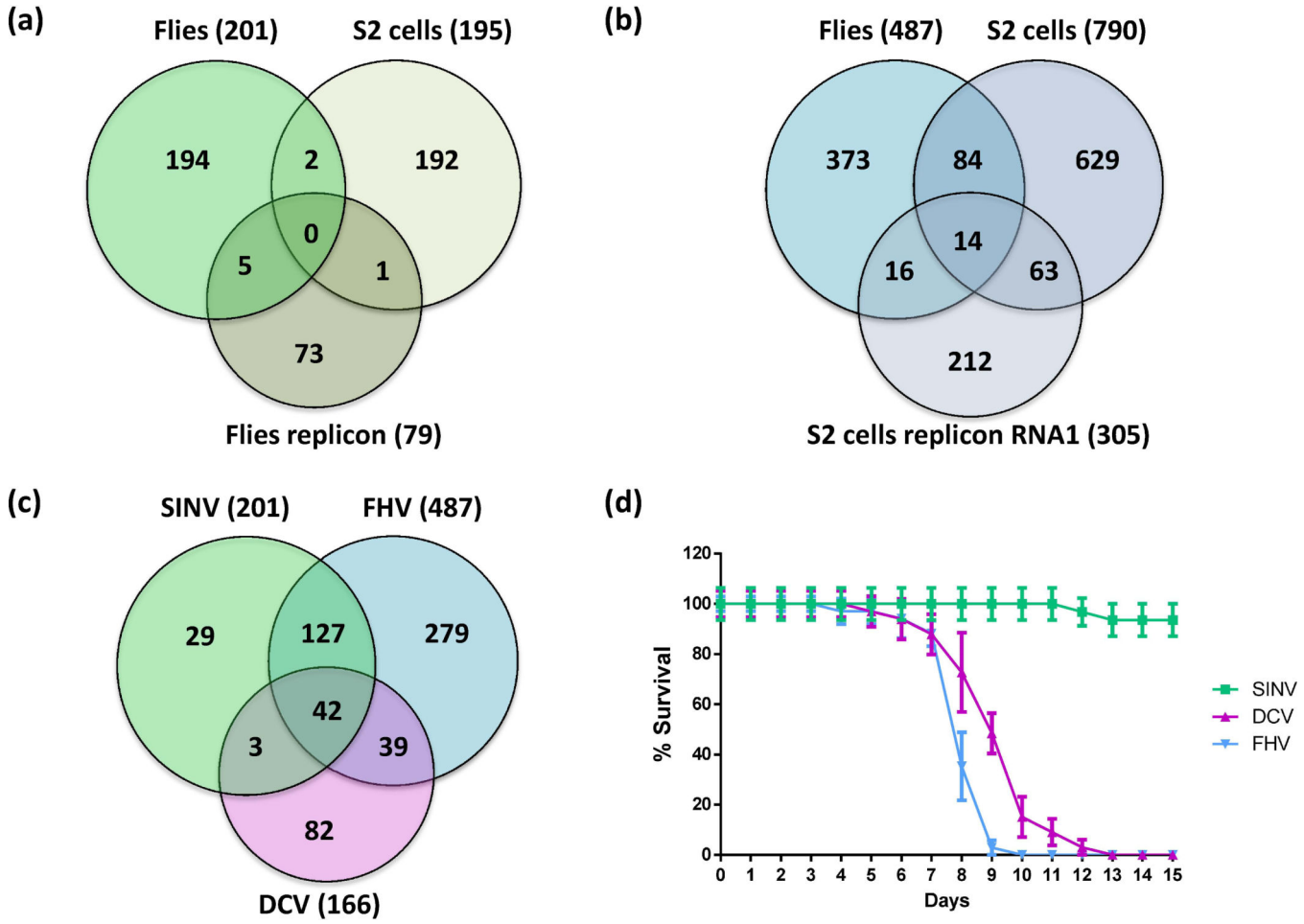

Figure 2. Inducible transcriptional responses to viral infection in *Drosophila*

(**a**) Venn diagram of genes induced during an infection by SINV in adult flies [16], in S2 cells [18] and adult flies expressing a SINV replicon [15]. (**b**) Venn diagram of genes induced during an infection by FHV in adult flies [16], in S2 cells and S2 cells expressing a RNA1 FHV replicon [17]. (**c**) Venn diagram of genes induced during an infection by SINV, FHV and DCV in wild type Oregon-R adult flies [16]. (**d**) Survival of wild type flies *Oregon-R* following infection by the indicated viruses. For panels **a-c**, published data were compared using GeneVenn (http://genevenn.sourceforge.net/). The total of genes induced in each condition is indicated in parenthesis. See supplementary Table 1 for details.