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Highlights

- siRNAs and miRNAs, but not piRNAs, regulate viral infection in *Drosophila*.
- NF- κ B mediated inducible responses contribute to antiviral immunity in flies.
- *Drosophila* can be used to decipher the ancestral role of STING in antiviral immunity.

Sensing and signalling viral infection in Drosophila

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Abstract:

The fruitfly *Drosophila melanogaster* is a valuable model to unravel mechanisms of innate immunity, in particular in the context of viral infections. RNA interference, and more specifically the small interfering RNA pathway, is a major component of antiviral immunity in drosophila. In addition, the contribution of inducible transcriptional responses to the control of viruses in drosophila and other invertebrates is increasingly recognized. In particular, the recent discovery of a STING-IKK β -Relish signalling cassette in drosophila has confirmed that NF- κ B transcription factors play an important role in the control of viral infections, in addition to bacterial and fungal infections. Here, we review recent developments in the field, which begin to shed light on the mechanisms involved in sensing of viral infections and in signalling leading to production of antiviral effectors.

Keywords:

NF- κ B; STING; RNA interference; innate immunity; antiviral factors; virus

1. Introduction

All living organisms face viral infections during their lifetime. To defend themselves, hosts developed various antiviral immune responses throughout evolution. In this regard, insects represent an interesting opportunity, in light of the fantastic biodiversity they represent, with over 1 million known species and an evolutionary history covering 350 million years (Misof et al., 2014). Historically, insect-virus interactions were first studied in the context of diseases affecting economically important insects, such as silkworms (Lü et al., 2018) or bees (Manley et al., 2015). Insect viruses also rapidly became of interest as biological control agents (e.g. baculoviruses (Lacey et al., 2015)). Finally, important animal and human viral diseases are transmitted by *Culex* and *Aedes* mosquitoes, e.g. West Nile, Yellow Fever, Dengue, Zika and Chikungunya (Shaw and Catteruccia, 2019). Pioneering studies in drosophila and genome sequencing have, since the early 2000s, promoted the expansion of the field of insect antiviral immunity.

Among insects, the fruit fly *Drosophila melanogaster* has a special status as an invaluable model organism, used since the beginning of the XXth century to understand the genetic basis of heredity, embryonic development as well as a range of biomedical problems, including immunity (reviewed in (Wangler et al., 2015)). Research on drosophila host defence against bacterial and fungal infections led to the identification of two evolutionarily conserved signalling pathways, Toll and Immune deficiency (IMD), regulating transcription factors of the NF- κ B family to induce production of antimicrobial peptides (AMPs; reviewed in (Hoffmann et al., 1999; Hultmark, 2003; Lemaitre and Hoffmann, 2007)). Cellular responses, driven by hemocytes, also participate in the control of infections (Gold and Brückner, 2015; Lamiable et al., 2016a; Letourneau et

al., 2016; Nainu et al., 2015; Weavers et al., 2016). More recently, the interest for antiviral resistance mechanisms in drosophila has grown, initially taking advantage of the pioneering work of Philippe Lhéritier and Nadine Plus who described the first drosophila viruses, Sigma virus and Drosophila C virus (DCV, Table 1 (Jousset et al., 1972; Lhéritier, 1958)). This led to the identification of the first genes encoding restriction factors (e.g. (Cao et al., 2017, 2016; Gay, 1978)), the description of induced responses (e.g. (Dostert et al., 2005; Kemp et al., 2013; Merkling et al., 2015b; Xu et al., 2012)), the discovery of the important role of RNA interference (RNAi ((Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006)) and the characterization of evolutionarily conserved host factors used by viruses or participating in the control of infections (e.g. (Eleftherianos et al., 2011; Majzoub et al., 2014; Panda et al., 2013)). In addition, the characterization of the drosophila virome led to the discovery of more than 20 new viruses, which represent a goldmine for future studies on virus-host interactions (Webster et al., 2015). Especially, the first natural DNA virus of *Drosophila melanogaster*, Kallithea virus (KV, Table 1), has been described and characterised (Palmer et al., 2018a).

Research carried on the genetics of drosophila antiviral immunity over the past 15 years led to the identification of many genes and pathways associated with host restriction factors, inducible responses and antiviral RNA interference (RNAi), which have been reviewed (Chow and Kagan, 2018; Liu et al., 2017; Mondotte and Saleh, 2018; Mussabekova et al., 2017; Palmer et al., 2018b; Segrist and Cherry, 2020; Swevers et al., 2018; Talide et al., 2020). Here, we focus on the recent advances in the field with a particular focus on the latest developments on RNA-based antiviral defences (RNAi) and on inducible responses. We also discuss remaining questions to better understand drosophila defences against viruses.

2. RNA-based antiviral immunity

RNAi, initially identified as a major antiviral pathway in plants, also plays an important role in drosophila immunity (reviewed in (Guo et al., 2019)). Small RNA silencing encompasses three different pathways in flies (Ghildiyal and Zamore, 2009), each of which participates in host-virus interaction, albeit at different levels. Two of these pathways involve small RNAs produced by Dicer (Dcr) enzymes, the 22-23nt long micro (mi) RNAs produced by Dcr-1 and the 21nt-long small interfering (si) RNAs produced by Dcr-2. Dicers are RNaseIII enzymes that process double-stranded (ds) RNA precursors into small RNA duplexes. In the case of miRNAs, a complex composed of another RNase III enzyme, Drosha, and a dsRNA binding protein (dsRBP), Pasha, processes the primary transcripts of mi-RNA (pri-miRNA) in the nucleus. After export to the cytoplasm, the precursor of the miRNA (pre-miRNA) containing a stem loop structure, is recognized and cleaved by Dcr-1 (Ha and Kim, 2014; Lee et al., 2004). In the case of Dcr-2, the template is a long dsRNA, which can result from either convergent transcription or the presence of long inverted repeats in a transcript (endo-siRNAs), but also from internalized dsRNAs (exo-siRNAs)(Czech et al., 2008; Kawamura et al., 2008; Marques et al., 2010) or intermediates of replication typically produced during a viral infection (vsiRNAs) (Aliyari et al., 2008; Bronkhorst et al., 2012; Mueller et al., 2010; Sabin et al., 2013; Vodovar et al., 2011). These small RNAs are then loaded onto enzymes of the Argonaute (AGO) family, AGO1 for miRNAs and AGO2 for siRNAs, where one of the strands will be discarded. The remaining strand, known as the guide strand, is used to direct the AGO enzyme towards its target RNA for translation inhibition or degradation, thus achieving silencing of gene expression. The third pathway involves slightly longer small RNAs, the 24-28nt

long PIWI-associated RNAs (piRNAs), which are produced by a Dcr-independent mechanism and associate with a different clade of AGO enzymes, known as PIWI (Saito et al., 2006; Vagin et al., 2006). The drosophila genome encodes three of these, Piwi, AGO3 and Aubergine (Senti and Brennecke, 2010; Siomi et al., 2011).

2.1. Activation of the siRNA pathway by viral and non viral RNAs

The multiple lines of evidence supporting a major role in antiviral defense for the siRNA pathway, including (i) the phenotypic characterization of flies mutant for the genes encoding the three core components of the pathway, *Dcr-2*, *r2d2* and *AGO2*; (ii) the identification of virus-derived siRNAs in infected flies; and (iii) the identification of viral suppressors in several insect viruses, have been reviewed elsewhere (Aguilar et al., 2016; Guo et al., 2019; Nayak et al., 2013; Schuster et al., 2019). The importance of the pressure put by viruses on the siRNA pathway to escape it is probably best illustrated by the viral suppressor of RNAi encoded by the Cricket Paralysis virus (CrPV, Table 1), CrPV-1A. This small viral protein targets AGO2 by two different mechanisms, directly blocking its RNA slicing activity and, at the same time, recruiting cellular proteins to assemble a virus-hijacked E3 ligase to destabilize the enzyme (Nayak et al., 2018; Watanabe et al., 2017). Intriguingly, CrPV-1A can also repress host transcription through a different domain, suggesting that this viral protein may also act as a suppressor of inducible responses (Khong et al., 2017).

Important remaining questions in the field pertain to the mechanism by which Dcr-2 recognizes viral RNA and the exact nature of the viral templates that are sensed by the enzyme. Interestingly, it was recently shown that Dcr-2 initiates cleavage of dsRNA differently depending on their extremities. Whereas the platform-PAZ domain recognizes templates with 3'OH overhang extremities, dsRNA with blunt termini

engage the N-terminal helicase domain (Sinha et al., 2018). This helicase domain belongs to the superfamily (SF) 2 group of helicases, like the retinoic acid-inducible gene (RIG)-I like receptors in vertebrates, which sense viral RNAs in the cytosol and trigger interferons (IFNs) synthesis. SF2 helicases exhibit three subdomains, Hel1, Hel2i and Hel2, which change conformation upon binding viral RNA, thus triggering signaling (in the case of RIG-I like receptors (Kolakofsky et al., 2012; Kowalinski et al., 2011)) or processive cleavage of dsRNA (in the case of Dcr-2 (Sinha et al., 2018, 2015)). This suggests an ancestral function for the helicase domain of Dcr-2 in antiviral immunity, associated to a role in recognition of non-self RNA. By contrast, the platform-PAZ domain, located downstream of the N-terminal helicase domain, may mediate recognition of self RNA and participate in the cellular regulatory functions of Dcr-2 (e.g. endo-siRNA pathway). Small dsRBPs, such as Loquacious (Loqs) and R2D2, play important roles in the modulation of the different activities of Dcr-2 (Cenik et al., 2011; Hansen et al., 2019; Marques et al., 2010; Trettin et al., 2017). These dsRBPs have two or three dsRNA binding motifs, which bind either dsRNA or, following loss of amino-acids important for nucleic acid binding, other proteins to regulate their activity. Interestingly, some of these dsRBPs interact with the helicase domain of Dcr-2 and modulate its functions (Donelick et al., 2020; Hansen et al., 2019). For example, the PD isoform of Loqs is essential for the generation of endo- and exo-siRNAs but dispensable for the production of vsiRNAs (Marques et al., 2013). This observation, confirmed by reconstitution experiments in human cells with drosophila genes (Girardi et al., 2015; Kennedy et al., 2017), reveals that cofactors of the core components Dcr-2, R2D2 and AGO2 play important roles in the siRNA pathway. Thus, Dcr-2 may be able to sense viral dsRNAs with blunt termini as non-self through its helicase domain, while requiring loqs-PD for the processing of endo siRNAs. Intriguingly, in *Aedes*

mosquitoes, which are important vectors for Dengue and other viruses, the *Loqs* gene has been duplicated and *Loqs2*, one of the two paralogues, is required for the antiviral siRNA pathway, but dispensable for the endo- and exo-siRNA pathways (Olmo et al., 2018). Overall, these results point to the importance of characterizing the termini of the dsRNAs processed by Dcr-2 in virus infected cells or flies to better understand its function. Identifying the exact role of Loqs and related proteins represents another frontier for the field in the coming years, all the more so because other small dsRBD proteins appear to be involved in RNAi. One of them is Blanks, a testis specific factor, which is required to export dsRNAs from the nucleus to the cytosol, where they can be processed by Dcr-2 (Nitschko et al., 2020). Interestingly, innate immunity genes are upregulated in the testis of *blanks* mutant flies, pointing to a possible connection between RNAi and other innate immunity pathways (Gerbasi et al., 2011). While Blanks does not seem to participate in antiviral immunity, another related protein, DISCO interacting protein 1 (DIP1), has been proposed to play a role in the control of viral infections although the mechanism remains unknown (Zhang et al., 2015).

Finally, antiviral RNAi in drosophila has also been reported to involve a systemic component, associated with reverse transcription of viral RNA into DNA and production of secondary siRNAs. The characterization of the specific components of systemic RNAi and the mechanisms involved represent an important challenge for the field (Goic et al., 2013; Mondotte et al., 2018; Poirier et al., 2018; Saleh et al., 2009; Tassetto et al., 2017).

2.2. Regulation of gene expression by Dcr-1 and Dcr-2 contributes to virus-host interaction in flies

Although primarily involved in the regulation of cellular gene expression, the miRNA pathway is also involved in host-virus interactions in many animals, including flies. For example, several DNA viruses produce miRNAs that modulate expression of host genes to facilitate dissemination of the virus (reviewed in (Tuddenham and Pfeffer, 2011)). The identification of an abundant miRNA, produced by KV, with up to few hundreds putative targets in the drosophila genome, paves the way to the characterization of this type of regulation in flies (Webster et al., 2015). In addition, some cellular miRNAs have been shown to modulate DCV replication by regulating proviral genes (e.g. miR-8-5p and dJun ((Monsanto-Hearne et al., 2017a)) or antiviral genes (e.g. miR956 and Ect4 ((Monsanto-Hearne et al., 2017b))).

Besides its role in RNA silencing, Dcr-2 also participates in non-canonical cytoplasmic poly-adenylation of a subset of messenger RNAs in drosophila embryos. Of note, at least two immunity related genes, Toll and R2D2, depend on Dcr-2 for their poly-adenylation (Coll et al., 2018). This study suggests that a complex composed of Dcr-2 and Wispy, a cytoplasmic poly(A) polymerase, is necessary for cytoplasmic poly-adenylation of these genes. This newly described function of Dcr-2, which affects the translation efficiency of the targeted genes, may help explain other roles of Dcr-2 that seem to be RNAi-independent. These include an involvement of Dcr-2 in resistance to various stresses, such as starvation, cold, but also oxidative or endoplasmic reticulum stresses (Lim et al., 2011). A direct post-transcriptional effect of Dcr-2, involving interaction between the helicase domain and the mRNA, may also regulate the expression of genes associated with host defense, e.g. the single von Willebrand factor-C (SVC) domain proteins like Vago in drosophila, which participate in antiviral immunity in flies, mosquitoes and bumblebees (Asad et al., 2018; Deddouche et al.,

208; Deng et al., 2020; Harsh et al., 2018; Paradkar et al., 2012; H. Wang et al., 2017).

2.3. *The piRNA pathway and the control of foreign genetic elements*

In drosophila, the piRNA pathway plays a crucial role in the protection of the germline against transposable elements (Brennecke et al., 2007; Pélisson et al., 2007; Senti and Brennecke, 2010). Ten years ago, piRNAs derived from viruses persistently infecting a drosophila cell line derived from ovaries (OSS cells) were reported, raising the possibility that the piRNA pathway participated in antiviral immunity in the germline and possibly also in somatic tissues (Wu et al., 2010). Rapidly, a role for this pathway was also proposed in *Aedes* mosquitoes, based on expansion of the Piwi family in these insects, some of which are clearly expressed in somatic tissue, and the identification of virus-derived piRNAs first in cell lines but also in mosquitoes for Chikungunya virus (reviewed in (Miesen et al., 2016)). In drosophila, subsequent *in vivo* experiments ruled out a role for the germline-specific piRNA pathway in antiviral defense, even in ovaries where the piRNA pathway is primarily active (Martins et al., 2019; Petit et al., 2016) and the biological relevance of the initial observation in the OSS cell line remains unclear. Thus, flies appear to be different from mosquitoes regarding a possible role of piRNAs in antiviral immunity. This observation highlights the need to proceed with caution when extrapolating results from drosophila to other insects, as further emphasized below.

A recent pan-arthropod metagenomic analysis revealed that somatic piRNAs targeting transposable elements are common among arthropods, unlike drosophila. The same study suggested that although some viruses may be targeted by somatic piRNAs in mosquitoes, the primary antiviral defense against viruses across arthropod

relies on siRNAs, as observed in flies (Lewis et al., 2018). Of note, the importance of the siRNA pathway in antiviral defense in drosophila and in the nematode *Caenorhabditis elegans*, two popular laboratory animal models, has led to the assumption that RNAi represented the default antiviral pathway in invertebrates, before the emergence of IFNs in vertebrates (tenOever, 2016). Interestingly, this view has been challenged lately as metagenomic sequencing analysis failed to identify canonical vsRNAs in most animals belonging to divergent phyla (Porifera, Cnidaria, Echinodermata, Mollusca, and Annelida) (Waldron et al., 2018). Even in drosophila, antiviral resistance mechanisms independent of RNA silencing are emerging in some tissues, e.g. epithelia (Martins et al., 2019; Mondotte et al., 2018; Palmer et al., 2020). Therefore, the prominent role of RNAi in antiviral immunity described for arthropods and nematodes may not be representative of the majority of invertebrates (Waldron et al., 2018).

3. Inducible responses to virus infection in flies

The recent discovery of IFN-like induced responses to virus infection in oysters (Lafont et al., 2020; Martins, 2020) and the conservation of the cGAS-STING pathway in the sea anemone *Nematostella vectensis* (Kranzusch et al., 2015) suggest that ancestral metazoan antiviral immunity involved induced expression of antiviral factors. Virus infection in drosophila is associated with important modifications of the host transcriptome (e.g. Dostert et al., 2005; Kemp et al., 2013; Merkling et al., 2015b; Xu et al., 2012). Some of these modifications reflects the altered physiology of infected flies. For example, DCV infection of the smooth muscle cells surrounding the crop, a food storage organ at the entry of the midgut, results in accumulation of peritrophic

matrix at the entry of the midgut and intestinal obstruction. This alters the physiology of the flies and results in strong repression of a large number of genes associated with digestion (e.g. Jonah proteases) upon DCV infection (Arnold et al., 2013; Chtarbanova et al., 2014). Viral infections are also associated with cellular and tissular stress (e.g. inhibition of cap-dependent translation by internal ribosome entry site (IRES)-containing viruses, release of cell debris upon cell lysis, accumulation of unfolded proteins). Such stress can result in gene induction, as suggested by the upregulation of heat-shock proteins upon viral infection (Merkling et al., 2015b). The first pathway reported to be associated with antiviral immunity in flies, the Jak/STAT pathway, highlights the difficulty to dissociate stress responses from *bona fide* immune mechanisms.

3.1. The Jak/STAT pathway and antiviral defense in drosophila

The Janus kinase/signal transducer and activator of transcription (Jak/STAT) signalling pathway is highly conserved in metazoan. In mammals, there are 4 Jak kinases and 7 STAT factors that mediate signalling downstream of receptors for many cytokines, including IFNs (Aaronson and Horvath, 2002). In drosophila, the pathway is composed of a single Jak kinase, Hopscotch (Hop (Hanratty and Dearolf, 1993; Perrimon and Mahowald, 1986)) and a single STAT factor STAT92E (Hou et al., 1996; Yan et al., 1996), acting downstream of the receptor Domeless (Dome), a homologue of the gp130 subunit of the receptors for cytokines of the interleukin-6 family (Brown et al., 2001). The drosophila genome encodes three cytokine-like Unpaired (Upd) proteins, which function as activating ligands for the Dome receptor (Harrison et al., 1998; Hombria et al., 2005; Wang et al., 2014). Initially characterized in drosophila for its developmental functions, the Jak/STAT pathway is also involved in tissue

homeostasis in adult flies (reviewed in (Zeidler and Bausek, 2013)). Whereas Upd1 appears to function mainly in development, Upd2 and 3 are induced by a number of stresses, including viral infection (Kemp et al., 2013; West and Silverman, 2018; Wright et al., 2011).

Analysis of the transcriptome of DCV-infected flies revealed induction of a number of genes enriched for STAT92E binding sites in their promoter regions. Mutational analysis of the promoter of the highly induced marker gene *vir-1* confirmed that these sites were essential for gene induction (Dostert et al., 2005). Genetic analysis confirmed that *vir-1* and a number of other genes are no longer induced by DCV infection in *Hop* mutant flies. Moreover, these flies are more sensitive to DCV and CrPV infection, although no phenotype was observed for other RNA or DNA viruses (Kemp et al., 2013). These results point to a role for Jak/STAT regulated genes in antiviral response against *Dicistroviridae*, even if antiviral effectors induced by the pathway have not yet been identified (Dostert et al., 2005b; Kemp et al., 2013, Figure 1). In another study, Merkling et al. reported a role for *G9a*, encoding a histone H3 lysine 9 methyltransferase, in the regulation of the Jak/STAT pathway (Merkling et al., 2015a). *G9a* mutant flies present a shortened lifespan upon infection with the RNA viruses DCV, CrPV, Flock House virus (FHV, Table 1) and Drosophila X virus (DXV, Table 1), which is not associated with increased viral load but rather with an hyperactivation of the Jak/STAT pathway. These results suggest that the epigenetic regulator G9a modulates the activation of the Jak/STAT pathway in response to infection by RNA viruses, in order to prevent deleterious effects caused by hyperactivation (Figure 1). Of note, the authors observed induction of *vir-1* and of the stress-induced *Turandot* genes, *TotA* and *TotM* (Ekengren et al., 2001), 24h after DCV, CrPV, DXV and FHV infections. In contrast, Kemp and collaborators did not detect

induction of *vir-1* upon DXV infection and only observed upregulation of *Tot* genes a few days after DCV infection, when the flies were starting to die (Kemp et al., 2013). These discrepancies may be explained by differences in the genetic background of the flies used, which could affect induction patterns of the *Turandot* and *vir-1* genes depending on the virus used for the infection, or environmental conditions of infection (e.g. dose of virus, fly media, microbiota).

The role of the Jak/STAT pathway is not limited to the response against RNA viruses, as it also controls the DNA virus Invertebrate iridescent virus (IIV)-6 (Table 1). IIV-6 infection significantly decreases the lifespan of STAT92E silenced flies (West and Silverman, 2018). Using S2 cells and flies carrying Upd hypomorphic alleles, these authors further demonstrated that IIV-6 infection induces expression of the three Upd proteins, which function redundantly to activate the Jak/STAT pathway. In these circumstances, the induction of the cytokines relies on the MAP-kinase p38b, one of the three drosophila p38 homologs. Accordingly, p38b mutant flies succumb more rapidly to IIV-6 infection than controls. West and Silverman further showed that induction of Upd expression required the NADPH oxidase NOX, suggesting that reactive oxygen species (ROS) production, resulting from the viral infection, was triggering activation of p38b. Of note, α -actinin, a component of the cortical cytoskeleton, which can be released upon tissue injury, was also shown to induce Upd3 in a NOX-dependent manner leading to the activation of the Jak/STAT pathway (Gordon et al., 2018; Srinivasan et al., 2016). Notably, signalling to Upd3 induction involved Shark and Src42A, the drosophila homologues of the mammalian Tyrosine kinases Syk and Src, which mediate induction of inflammation and adaptative immunity upon sensing actin, another cortical component of the cytoskeleton. Altogether, these data suggest that release of α -actinin upon IIV-6 triggered cell death induces

production of Upd cytokines, leading to the activation of the Jak/STAT pathway (Figure 1).

In summary, the Jak/STAT pathway seems to be induced primarily as a result of cell damage caused by viral infection, rather than by directly sensing viral molecular patterns in drosophila. In addition, this pathway appears to primarily increase resilience to infection, rather than resistance, as mutant flies exhibit increased lethality, with little or no impact on viral titres (Merkling et al., 2015a; West and Silverman, 2018). Alternatively, the viruses tested may have evolved suppressor mechanisms opposing antiviral effectors induced by the Jak/STAT pathway. In this regard, it is interesting to note that infection of S2 cells by the newly discovered DNA virus KV resulted in downregulation of a reporter gene driven by a synthetic promoter composed of 10 STAT-binding sites (Palmer et al., 2019). This suggests that KV encodes a suppressor, the characterization of which would provide strong evidence for the importance of the Jak/STAT pathway in antiviral immunity in flies.

3.2 Control of viral infections by the Toll pathway

Two distinct pathways, Toll and IMD, rely on NF- κ B transcription factors to regulate expression of AMPs genes in drosophila ((Buchon et al., 2014) and references therein). Toll regulates Dorsal (DI) and Dorsal-related immunity factor (DIF), whereas IMD controls the third member of the family in drosophila, Relish. These pathways have well established roles in the control of bacterial and fungal infections but involvement in antiviral defence has also been suggested (Figure 1).

A comprehensive study by Teixeira and collaborators reported the involvement of the Toll pathway in the defence against a broad range of RNA viruses (DCV, CrPV, FHV, Nora virus (Table 1)(Ferreira et al., 2014)). Interestingly, the authors observed

that the Toll pathway is important to survive oral infection but not systemic infection by these viruses, even though activation of the Toll pathway is detected in the fat body following both types of infection. This emphasizes the importance of the route of infection for host-pathogen interaction (Martins et al., 2013). Furthermore, the data suggest that the Toll pathway is targeting a step of the viral cycle occurring specifically in the course of oral infection and bypassed by an injection directly in the hemocoel (Ferreira et al., 2014). Of note, in addition to *spaetzle*, *Toll* and *pelle*, *dorsal* mutants presented an increased susceptibility to oral infection while *Dif* mutant did not. These results suggest that, while DIF mediates the Toll-dependent response triggered by bacteria and fungi infection (Rutschmann et al., 2000), DI is responsible for antiviral defences (Ferreira et al., 2014), providing an explanation for the long-noted expression of DI in the fat body of adult flies (Lemaitre et al., 1995).

Obbard and colleagues did not observe increased sensitivity of Toll pathway mutant flies to infection with the DNA virus KV, which may be explained by the fact that they did not use an oral infection model. However, they reported a strong suppression of Toll pathway activity in KV infected flies (Palmer et al., 2019). They further identified a previously uncharacterized KV gene, gp83, which encodes a potent suppressor of Toll signalling (Figure 1). The mode of action of this nuclear protein, which appears to act downstream of DIF or DI, is still mysterious. Whatever the mechanism, identification of a viral suppressor provides further evidence for a role of the Toll pathway in antiviral immunity.

Overall, these findings raise the question of the mechanism by which viral infection may activate the Toll pathway. One possibility is that tissue damage and necrosis caused by viral infection may result in activation of the Toll pathway through activation of the Persephone (*psh*) protease (Figure 1 (Ming et al., 2014; Tang et al.,

2008)). Another important question pertains to the induction of an antiviral versus antifungal response by the Toll pathway. While it could be argued that DIF and DI may regulate antifungal and antiviral effector genes, respectively, it is intriguing that some reports suggest an involvement of DIF, rather than DI, in antiviral immunity, independently of the canonical Toll pathway. Indeed, *dif*, but not *spz*, *tll*, *tube* and *pelle*, mutant flies were reported to exhibit increased sensitivity to DXV (Zambon et al., 2005). Although the genetic background was not controlled in these early experiments, it is intriguing that induction of the cytokine Dieldel (Die) following Sindbis virus (SINV, Table 1) infection depends on DIF but not MyD88, which is the adaptor of the canonical Toll pathway (Lamiabile et al., 2016b, Figure 1). Finally, a recent report points to the activation of a non-canonical pathway leading to expression of two Toll-regulated AMPs, drosomycin and attacin-A in response to DCV infection (Zhang et al., 2020). This highlights the importance of further investigations on the role of the Toll pathway in antiviral immunity, and on the respective contributions of DIF and DI in the expression of genes involved in antiviral defenses.

3.3 Relish-dependent antiviral immunity

A role of the IMD pathway in the resistance to CrPV and SINV was first reported in 2009 (Avadhanula et al., 2009; Costa et al., 2009), followed a few years later by the description of antiviral activity for two antimicrobial peptides, Diptericin-B and Attacin-C (Huang et al., 2013). It is interesting to note that, besides its role in the expression of antibacterial peptides, the IMD pathway is also implicated in the regulation of apoptosis (Georgel et al., 2001; Tavignot et al., 2017; Zhai et al., 2018). Indeed, programmed cell death represents an efficient antiviral defense, interrupting viral replication (Liu et al., 2013). Efficient phagocytosis of apoptotic cells further prevents

409 dissemination of viruses (Lamiable et al., 2016a; Nainu et al., 2015). In drosophila,
410 apoptosis is largely controlled at the level of the inhibitory protein DIAP1, which
411 prevents activation of caspases. The IMD pathway may regulate apoptosis in the
412 context of viral infection by triggering DIAP1 protein degradation via DIAP2-mediated
413 polyubiquitination (Herman-Bachinsky et al., 2007). However, the situation is likely to
414 be more complex as IMD signalling antagonizes apoptosis in some tissues during
415 development by upregulating *DIAP1* gene transcription (Tavignot et al., 2017). Of note,
416 DCV was shown to suppress the N-end rule pathway, one of the mechanisms causing
417 DIAP1 degradation. This enables the virus to block or slow down programmed cell
418 death resulting in enhanced replication (Wang Z. et al., 2017).

419 Characterization of the virus-induced gene *Die* confirmed a role of the IMD
420 pathway in antiviral immunity (Lamiable et al., 2016b). Indeed, *Die* mutant flies were
421 more sensitive to infection with SINV than controls and early lethality was not
422 accompanied by increased viral titer but rather correlated with strong upregulation of
423 IMD target genes. Flies double mutant for *Die* and *IMD* or *ikk γ* resisted like wild-type
424 flies to SINV infection, confirming that it was the overactivation of the IMD pathway,
425 rather than the lack of *Die per se*, that caused the demise of the flies. *Die* encodes a
426 small circulating protein that probably functions as an immunomodulatory cytokine
427 downregulating the IMD pathway by an unknown mechanism (Lamiable et al., 2016b).
428 Intriguingly, this cytokine also appears to inhibit apoptosis in developmental contexts
429 (Mlih et al., 2018). *Die*-related genes can be found in the genomes of DNA viruses
430 belonging to at least three families (Ascoviridae, Entomopoxviridae, Baculoviridae,
431 Figure 1), and at least one of them can rescue the phenotype of *Die*-mutant flies
432 (Lamiable et al., 2016b). This result indicates that insect viruses have, on several
433 occasions, hijacked a suppressor gene of the IMD pathway, suggesting that activation

of this pathway during infection exerts a pressure on viruses. Interestingly, IIV-6 does not encode a Die homologue but expresses another as yet poorly characterized suppressor of IMD signalling, acting in the nucleus at the level of Relish-mediated transcriptional activation (West et al., 2019).

Together with previous results reporting inhibition of NF- κ B signalling by vankyrin proteins encoded by polydnviruses (Gueguen et al., 2013), these recent findings prompted a careful examination of the role of the IMD pathway in the control of viral infection in the S2 cell line. This revealed that silencing of IKK β and Relish resulted in significantly increased replication of DCV. Unexpectedly, silencing of the other genes of the IMD pathway did not affect viral load, with the exception of IKK γ /NEMO, the regulatory subunit of the IKK complex, for which viral replication was decreased (Goto et al., 2018). This suggested that downstream components of the pathway could be activated independently of IMD in response to a viral infection. One of the genes induced by DCV infection in an IKK β and Relish-dependent manner is the homologue of the gene STING (stimulator of interferon genes). In mammals, STING is a key component of the cytosolic DNA sensing system, activating IFN genes in response to a second messenger, the cyclic dinucleotide (CDN) 2'3'-cyclic GMP-AMP (2'3'-cGAMP). This CDN contains one 2',5' phosphodiester bond joining G to A and one canonical 3',5'-phosphodiester bond joining A to G. It is produced by the enzyme cyclic GMP-AMP synthase (cGAS), which becomes activated upon binding DNA or DNA/RNA duplexes in the cytosol (reviewed in (Ablasser and Chen, 2019)). Of note, STING can also be activated by CDNs of bacterial origin, which contain two canonical 3',5' phosphodiester-linkages (3'3'-cGAMP, c-di-GMP, c-di-AMP (Margolis et al., 2017)). In drosophila cells, ectopic expression of dSTING can prevent replication of DCV in an IKK β - and Relish-dependent manner, suggesting that dSTING acts

upstream of these two proteins in a new pathway activated by viral infections (Goto et al., 2018). Interestingly, similar results were reported in *Bombyx mori*, where a homologue of STING activates Relish and antiviral immunity in response to infection by nucleopolyhedrovirus (NPV (Hua et al., 2018)). Altogether, these results suggest that an ancestral function of the evolutionarily ancient molecule STING is the regulation of NF- κ B-dependent transcription of antiviral genes, predating the appearance of IFNs (Figure 1). Of note, activation of Relish by the IMD pathway in response to bacterial infections requires both its phosphorylation by IKK β and its cleavage by the caspase DREDD in a process that involves the IKK γ /NEMO protein (reviewed in (Buchon et al., 2014)). By contrast, activation of Relish by dSTING does not appear to require DREDD and is opposed by IKK γ /NEMO (Goto et al., 2018).

It is noteworthy that an IMD-independent function of Relish has previously been described in salivary gland degradation during metamorphosis (Nandy et al., 2018). Thus, besides the IMD pathway, Relish can be regulated by non-classical pathways and activate transcriptional outputs distinct from the IMD pathway, probably in association with specific transcription cofactors (Zhai et al., 2018). Interestingly, Relish regulates expression of the gene *Atg1* in salivary glands to trigger autophagy-dependent cell death. This may provide a connection with the role of dSTING in antiviral immunity. Indeed, in mammals, besides expression of IFNs, STING also regulates autophagy, which is thought to represent an ancestral antiviral function (Gui et al., 2019; Margolis et al., 2017). Similarly, dSTING was reported to induce autophagy in the drosophila brain to control ZIKA virus replication (Liu et al., 2018).

A major question at this point pertains to the activation of Relish in virus-infected flies. In the absence of bacterial infection, the IMD pathway may be activated by a response to necrotic cells possibly driven by dysbiosis (Kosakamoto et al., 2020) or by

deregulation of the expression of the receptors PGRP-LC or -LF (Nandy et al., 2018; Tavignot et al., 2017). The involvement of dSTING in the regulation of Relish activity in the context of viral infections now raises the question of the mechanisms by which it gets activated. The drosophila genome encodes one obvious cGAS homologue, CG7194, which does not seem to participate in antimicrobial defenses (Martin et al., 2018; Wu et al., 2014). This led Goodman and colleagues to propose that in drosophila, dSTING is activated by bacterial CDNs, rather than the host-produced second messenger 2'3'-cGAMP. However, we recently observed that 2'3'-cGAMP is a more potent activator of STING-regulated genes in drosophila than 3'3'-linked bacterial CDNs (Cai et al., 2020). This suggests that a cGAS-like enzyme might exist in drosophila, as already reported in the sea anemone *Nematostella vectensis* (Kranzusch et al., 2015) and in the Lepidopteran insect *B. mori* (Hua et al., 2018).

4. Concluding remarks

Recent work in the field of drosophila antiviral immunity, while improving our understanding on the contribution of small regulatory RNAs, have provided increasing evidence that induced immune responses, and in particular NF- κ B pathways, also play an important role. Interestingly, inducible antiviral immune responses appear to play an important role in other invertebrates as well (e.g. (Lafont et al., 2017)). While initial studies suggested that invertebrates primarily relied on ancestral RNAi response to control viruses, which was replaced by IFN-regulated inducible responses in vertebrates, a more complex picture is now emerging. In particular, it is now clear that inducible antiviral immune responses predate the appearance of IFNs in vertebrates, and that some antiviral genes (e.g. cGAS, viperin, STING) were inherited from

procaryotes (Bernheim et al., 2020; Cohen et al., 2019; Morehouse et al., 2020; Sen and Peters, 2007). Of note, important differences among insects are also becoming apparent (e.g. presence of virus-derived piRNAs in mosquitoes but not in drosophila; absence of the STING gene in mosquitoes). These differences are not surprising in light of the strong pressures exerted on host genomes by rapidly mutating viruses. Documenting these differences and their analysis can reveal important information on the mechanisms at play in the arms race between viruses and the immune system of their hosts (Daugherty and Malik, 2012). In summary, while *D. melanogaster* will continue to be an invaluable tool in future work to decipher the complex host-virus interactions (e.g. molecular mechanisms of pathogenesis (Chow et al., 2017)), caution should be exerted before extrapolating findings in this model to other insects, all the more so to invertebrates in general.

The basic mechanisms involved in the restriction of viruses by the siRNA pathway in drosophila have now been well characterized, although key questions remain. Among them, the question of the sensing of viral RNAs is central, as it is now clear that there is more to it than the double strandedness of replication intermediates, for example. Future work will need to determine if the discrimination between viral and self RNAs involves the quantity of template for Dcr-2, differences at the extremities of the RNAs or their subcellular localization, all of which contribute to the activation of nucleic acid sensing pattern recognition receptors (PRRs) in mammalian cells (Roers et al., 2016). Clarification of the role of dsRBPs cofactors represents another priority for the field, together with the elucidation of the mechanism by which an intrinsically cell autonomous process (Roignant et al., 2003) can generate a systemic response in the context of viral infections (Saleh et al., 2009; Tassetto et al., 2017).

Regarding the emerging role of induced responses, the key question for future work relates to the identification of the receptors sensing viral infections, in particular whether these responses involve *bona fide* PRRs, and in this case what types of molecular patterns are recognized. The field will also greatly benefit from the investigation of the modes of action of viral suppressors of inducible responses, which could reveal new regulatory facets of inducible immunity pathways. The interplay of the IMD- and dSTING-dependent Relish functions in response to bacterial and viral infections, respectively, also deserves attention, as highlighted by the contribution of the microbiota to antiviral immunity (Sansone et al., 2015; Teixeira et al., 2008; Xu et al., 2013). Last but not least, a major unsolved question is the function of the many induced genes, which contribute to the control of viral infections. Indeed, the functional characterization of new antiviral factors could reveal original targets for the design of therapy against families of viruses infecting both insects and mammals, such as picorna-like viruses, alphaviruses, rhabdoviruses or poxviruses (Shi et al., 2016).

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Figure 1. Inducible antiviral responses in drosophila. The main signalling pathways associated with antiviral immunity in *D. melanogaster* are shown. Some responses are thought to be induced in infected cells, upon sensing infection (e.g. STING pathway), while others occur in response to cytokines (e.g. Dieder, Spätzle, Upd). Infected dying cells can release cellular components (e.g. α -actinin), detected as danger signals in a paracrine manner by other cells. Viral suppressors targeting these pathways are shown as orange triangles. See the text for details.

Figure 1

Figure1 Review JS and JLI

Name	Family	Genome	Identified immune suppressor		
			Name	Targeted pathway	Mechanism
Drosophila C virus (DCV)	<i>Dicistroviridae</i>	ssRNA(+)	DCV-1A	siRNA	dsRNA binding protein: long dsRNA
Cricket Paralysis virus (CrPV)	<i>Dicistroviridae</i>	ssRNA(+)	CrPV-1A	siRNA; transcription	AGO2 binding: inhibition & degradation
Sindbis virus (SINV)	<i>Togaviridae</i>	ssRNA(+)	/	/	/
Flock House virus (FHV)	<i>Nodaviridae</i>	ssRNA(+)	FHV-B2	siRNA	dsRNA binding protein: long dsRNA & siRNA duplexes
Nora virus	Unclassified	ssRNA(+)	Nora-VP1	siRNA	AGO2 binding: inhibition
Sigma virus (SV)	<i>Rhabdoviridae</i>	ssRNA(-)	/	/	/
Drosophila X virus (DXV)	<i>Birnaviridae</i>	dsRNA	DXV-VP3	siRNA	dsRNA binding protein: long dsRNA & siRNA duplexes
Kallithea virus (KV)	<i>Nudiviridae</i>	dsDNA	gp83	Toll	NF- κ B transcription factors
			?	Jak-STAT?	?
Invertebrate Iridescent virus 6 (IIV-6)	<i>Iridoviridae</i>	dsDNA	IIV6-340R	siRNA	dsRNA binding protein: long dsRNA & siRNA duplexes
			?	IMD/Toll	Relish-mediated transcriptional activation/?

Table 1: Characteristics of the viruses mentioned in the text.