The role of micro RNAs (miRNAs) in the regulation of Drosophila melanogaster’s innate immunity

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ABSTRACT
MicroRNAs (miRNAs) are a class of small non-coding RNAs ~19–22 nt long which post-transcriptionally regulate gene expression. Their ability to exhibit dynamic expression patterns coupled with their wide variety of targets allows miRNAs to regulate many processes, including the innate immune response of Drosophila melanogaster. Recent studies have identified miRNAs in Drosophila which are differentially expressed during infection with different pathogens as well as miRNAs that may affect immune signalling when differentially expressed. This review provides an overview of miRNAs which have been identified to play a role in the immune response of Drosophila through targeting of the Toll and IMD signalling pathways and other immune processes. It will also explore the role of miRNAs in fine-tuning the immune response in Drosophila and highlight current gaps in knowledge regarding the role of miRNAs in immunity and areas for further research.

Introduction
Innate immunity acts as the first line of host defence against pathogenic microorganisms. Proper regulation of associated innate immune responses is vital, as sufficiently potent activation is necessary to protect against invading pathogens, while it is also important to prevent overactive immune responses that could result in tissue damage [1].

Lacking an adaptive immune system, the effects of innate immunity can be easily isolated in the fruit fly Drosophila melanogaster, making it an attractive model organism to study innate immunity. The two Drosophila Nuclear Factor kappa B (NF-κB) pathways Toll and Immune deficiency (IMD) (Figures 1 and 2) are evolutionarily conserved and homologous to the mammalian Toll-like receptor (TLR)/Interleukin-1 Receptor (IL-1 R) and Tumour Necrosis Factor Receptor (TNFR) pathways, respectively [2,3]. In Drosophila, these pathways lead to the secretion of a variety of antimicrobial peptides (AMPs) in response to bacterial and fungal infections and have also been implicated in antiviral immunity [4,5]. Expression of the antifungal peptide Drosomycin (Drs) is primarily regulated by the Toll pathway, while expression of the peptide Diptericin (Dpt), which defends against Gram-negative bacteria, is primarily regulated by the IMD pathway [6], allowing expression of the genes encoding for these peptides to be used as measures of Toll and IMD signalling activation respectively. While largely independent, cross-regulation does occur between the Toll and IMD pathways [7]. Among AMPs whose expression depends on both pathways we find AttacinA (AttA) and Cecropin that act against Gram-negative bacteria, Defensin that acts against Gram-positive bacteria, and Metchnikowin (Mtk) which is an antifungal peptide [7,8].

Accumulating evidence over the past decade suggests the ability of microRNAs (miRNAs), to regulate innate immunity in Drosophila melanogaster. miRNAs are a group of endogenous, small non-coding RNAs ~19–22 nt long which post-transcriptionally repress gene expression through complementary base pairing between the seed region (nt 2–7) of the miRNA and the 3’ UTR.
of target mRNA [9]. Such repression occurs either via a mechanism that directly degrades the mRNA or an alternate mechanism that simply induces its decay [10]. Due to the short length of the seed region, each gene is capable of being regulated by multiple miRNAs, and each miRNA is capable of regulating a diverse set of hundreds of transcripts, with miRNAs being estimated to target around 30% of all *Drosophila* genes [11,12].
miRNAs are implicated in many biological processes, including innate immunity. Their biogenesis begins in the nucleus with transcription of primary miRNAs (pri-miRNAs) by RNA Polymerase II [13,14]. Pri-miRNAs are subsequently processed by a complex involving the nuclear RNase III Drosha and its co-factor Pasha to produce a ~60–70 nt stem-loop structure known as a precursor miRNA (pre-miRNA). The pre-miRNA is then exported to the cytoplasm upon formation of a complex involving the nuclear RNase III Drosha and its co-factor Pasha to produce a ~60–70 nt stem-loop structure known as a precursor miRNA (pre-miRNA). The pre-miRNA is then exported to the cytoplasm upon formation of a complex.
consisting of the pre-miRNA, the Exportin 5 protein, and its co-factor Ran-GTP [15–17]. Further processing by the RNase III enzyme Dicer-1 into a double-stranded duplex results in two potentially functional miRNAs [18] termed the 3p and 5p species based on whether they are derived from the 3’ or 5’ arm respectively [19]. Subsequent loading into a member of the Argonaute (AGO) family of proteins, primarily AGO1 but also AGO2 [20], forms an RNA-induced silencing complex (RISC) which then acts to silence its targets [21,22]. Drosophila with disrupted miRNA biogenesis pathways are more vulnerable to infection with Candida albicans (C. albicans) and experience differential expression of AMPs when infected with C. albicans and Escherichia coli (E. coli) [23,24]. This indicates that miRNA processing is an important mechanism in protecting flies against infections and suggests a role in innate immunity of these regulatory RNAs. Drosophila represent an excellent model for investigating miRNA-mediated regulation of innate immunity given their low cost of culture, short lifespan and developmental time, large number of offspring, and a small, easily manipulated genome. The binary Gal4/UAS expression system can be used in Drosophila for tissue and time-specific overexpression or knockdown of genes, including miRNAs [25,26]. There also exist a wide variety of tools for evaluating the effects of miRNA deficiency in vivo, including miRNA deletion mutants and miRNA-sponge mutants that express an oligonucleotide sponge construct that inhibits miRNAs via complementary binding [27,28]. Several miRNA target prediction algorithms have also been developed, including TargetScan, miRanda, PicTar, RNA22, and PITA [28]. Although these algorithms may sometimes provide false-positive results regarding miRNA–gene regulatory relationships, the application of various screening strategies can aid in the identification of relevant miRNA–gene interactions [29]. In vitro cell culture-based techniques such as luciferase reporter assays can also be applied. These reporters consist of fusing the predicted miRNA target sequence of the gene of interest (most often located in the 3’-untranslated region (3’-UTR) of the corresponding transcript) with the luciferase gene [30]. miRBase (www.mirbase.org), a database containing 258 miRNAs of Drosophila melanogaster in the latest version (v22.1) also provides an abundance of information on miRNAs, including annotations of potential miRNA targets, sequences, references in literature, deep sequencing data, and genome coordinates. Several other technologies are also used for the detection and quantification of miRNAs, including transcriptional reporters, in situ hybridization, northern blotting, quantitative real-time PCR (qRT-PCR), microarrays, and small RNA-sequencing [31]. This review will focus on the role and mechanisms by which microRNAs regulate innate immunity in Drosophila melanogaster.

Regulation of Drosophila Immune Deficiency (IMD) pathway by miRNAs

miRNAs are able to regulate innate immune responses mediated by the IMD pathway, which primarily responds to Gram-negative bacterial infections (Figure 1) [3]. IMD signalling is initiated when peptidoglycan receptor proteins recognize meso-diaminopimelic acid (DAP)-type peptidoglycan found in most Gram-negative bacteria [32–35], leading to formation of an intracellular complex involving the immune deficiency (Imd) protein, the adaptor protein dFadd and the caspase Dredd [3,36–39]. The formation of this complex results in the activation of the TGF-β activated kinase 1 (Tak1)/Tak1-associated binding protein 2 (Tab2) complex [3,40,41], which works to activate the IKK kinase complex composed of the proteins Kenny (Key) and Ird5 [41–44]. The active IKK complex then phosphorylates the NF-κB transcription factor Relish (Rel) resulting in its cleavage into Rel-68 and Rel-49 subunits [45], allowing Rel-68 to translocate to the nucleus and promote transcription of its target genes of the IMD pathway including AMPs [46].

In silico analysis indicated the ability of the miRNA let-7 to directly repress the IMD pathway AMP Dpt. This was validated using a luciferase reporter assay in cultured S2 cells [47], a cell line derived from embryonic macrophage-like cells [48]. In S2 cells, the steroid hormone 20-hydroxyecdysone (ecdysone) is capable of inducing let-7 signalling through an Ecdysone receptor (EcR) and Broad Complex (Br) dependent pathway [47,49,50]. The immediacy of such a transcriptional response is highly dependent on
prior priming and exposure to ecdysone [47], thus explaining why in experiments without priming, ecdysone signalling has a significantly delayed effect on let-7 transcription [49,51]. Surprisingly, in S2 cells, while ecdysone induces let-7 expression, it also induces expression of Dpt, a target that let-7 represses. Indeed, ecdysone has been previously shown to enhance AMP gene expression, including Dpt expression, following pathogen challenge by regulating the IMD pathway in both cultured cells and in vivo [52–56]. This led Garbuzov and Tatar, 2010 to suggest a model in which ecdysone induces Dpt expression but let-7 acts to prevent Dpt hyperactivation [47].

A separate report confirmed the effect EcR and Br have on mediating ecdysone signalling and the ability of such signalling to repress transcription of miR-34 [24]. EcR, Br, and the transcription factors Serpent (Srp), Twist (Twi), and Apterous (Ap) were shown to mediate ecdysone repression of miR-34. A number of cis-regulatory elements near the miR-34 locus, one of which is a Br target, also appear to be involved. Ecdysone represses miR-34 transcription, but miR-34 also represses br of the ecdysone signalling pathway, suggesting a positive feedback loop on miR-34 transcription constituted by ecdysone.

miR-34 acts as a positive regulator of IMD signalling. In vivo, ubiquitous miR-34 overexpression results in increased levels of Dpt transcripts in both the presence or absence of E. coli infection [24]. In vitro, in comparison to controls, ecdysone-treated S2 cells overexpressing miR-34 exhibit upregulated expression of several components of the IMD signalling pathway in both the presence and absence of peptidoglycan treatment. Among these, we find the AMPs Dpt, Cecropin A1 (CecA1), Atta, Defensin (Def) and Mtk, and the poor Imd response upon knock-in (pirk) mRNA. Silencing of IMD pathway components in miR-34 overexpressing S2 cells treated with ecdysone is associated with significantly decreased Dpt levels, indicating that miR-34-mediated modulation of Dpt expression occurs at least partially through the IMD pathway. Of note is the fact that ecdysone-treated S2 cells overexpressing miR-34 require peptidoglycan-recognition protein LC (PGRP-LC) to exert their immune-stimulating effects, even in the absence of peptidoglycan treatment. Due to the manner in which ecdysone is able to trigger PGRP-LC expression [53], it is possible that miR-34 mediates PGRP-LC expression in ecdysone signalling. Experiments involving miR-34 ubiquitously overexpressing and miR-34 knockout flies infected with pathogens including Gram-negative Erwinia carotovora carotovora strain 15 (Ecc15), Enterobacter cloacae (E. cloacae) and E. coli revealed that miR-34 enhances immune responses by promoting increased AMP expression and pathogen resistance independently of phagocytic activity [24]. Additional in silico, in vitro, and in vivo analysis showed that the immune regulatory effects of miR-34 are in-part due to direct repression of the genes discs large 1 (dlg1), Su(Z)12, CG8468, murashka (mura), Ecdysone-induced protein 74EF (Eip74EF) and Ecdysone-induced protein 75b (Eip75B).

The diverse targets of miR-34 signalling complicate our understanding of the exact mechanisms by which they regulate immunity. While the miR-34 targets Eip75B,dlg1, CG8468, mura and Su(Z)12 act as negative regulators of IMD signalling [24,40], the miR-34 target Eip74EF acts as a positive regulator of IMD signalling [53]. It is possible that miR-34 targets different genes during various stages of infection; however, more work is necessary to evaluate the temporal expression of its target genes during infection. Conflicting reports exist regarding changes in miR-34 expression in response to Gram-negative E. coli infection. One report found no changes in expression from 0 to 120 hours post infection [24], and another study identified decreased expression at 24 hours post infection [57], making the role of miR-34 in defending against acute E. coli infection unclear.

miR-34 belongs to a cluster in the genome that also includes miR-277, a negative regulator of the IMD pathway that is repressible via ecdysone signalling [24,58]. However, the relationship between ecdysone and IMD signalling regulated via miR-277 has not been well explored. One report identified that following E. coli infection, the Drosophila transcription factor Myc (dMyc) negatively regulates Dpt expression and IMD signalling through a pathway mediated by miR-277 [58]. A chromatin immunoprecipitation (ChIP)-qPCR assay showed that dMyc activates miR-277 expression via direct binding to its promoter, and
a luciferase reporter assay in S2 cells demonstrated that miR-277 directly targets imd and the Tab2-Ra/Rb isoforms whose products positively regulate IMD signalling [39,40]. In vivo, ubiquitous miR-277 overexpression downregulates both imd and Tab2 expression and can be rescued by the ubiquitous co-overexpression of a miR-277 sponge construct [58]. In the presence of E. coli infection, the downregulated Dpt expression in miR-277 overexpressing flies can also be rescued by co-overexpression of the miR-277 sponge construct. Phenotypically similar to the miR-277 overexpressing line is a dMyc ubiquitously overexpressing line that displays decreased expression of Dpt, imd, and Tab2 after E. coli infection. The expression of these genes is rescued in a dMyc and miR-277 sponge ubiquitously co-overexpressing line [58]. Examination of temporal patterns of gene expression in wild-type flies infected with E. coli revealed that dMyc and miR-277 exhibit opposite expression patterns from imd and Tab2, suggesting their ability to negatively regulate imd and Tab2. dMyc and miR-277 display an initial drop in expression after infection but become upregulated in the later stages of infection. This suggests they may initially become downregulated to allow for an adequately potent immune response but become upregulated in the later stages of infection to prevent overactivation of the immune system and restore immune homoeostasis. However, despite dMyc’s role as a negative regulator of the IMD pathway, dMyc overexpressing flies show enhanced survival compared to controls in response to E. cloacae infection [58]. It is possible that dMyc may have other immunomodulatory roles independent of its effects on miR-277 expression, which are responsible for such enhanced survival; however, further studies are needed to explore this possibility.

Investigation of the miR-959-964 cluster, which undergoes circadian cycling, revealed that these miRNAs act as negative regulators of immune function [59]. In vivo experiments using miR-959-962 knockout mutants (miR-963 and miR-964 were not knocked out) and ubiquitously overexpressing miR-959-964 flies, showed that the cluster negatively affects resistance to pathogenic Gram-negative Pseudomonas aeruginosa (P. aeruginosa) infection. Indeed, miR-959-962 knockout mutants infected with P. aeruginosa display varying circadian time-dependent survival, implicating cycling miR-959-962 levels in the time-dependent regulation of immunity. The role of immunity for this miRNA cluster is supported by the fact that the fat body, a major organ in immune function [60], is a prominent site of miR-959-964 cluster expression [59].

miR-9a and miR-981, two miRNAs upregulated in response to E. coli infection, also act as negative regulators of the IMD pathway [57]. Compared to controls, fly lines ubiquitously overexpressing miR-9a and miR-981, respectively, exhibit decreased levels of Dpt in response to E. coli infection. In these lines, the Dpt expression is rescued by ubiquitous co-overexpression of each miRNA’s sponge. In silico predictions and luciferase reporter assay in S2 cells validated the ability of miR-9a and miR-981 to directly target Dpt transcripts. miR-9a overexpressing flies also exhibit increased AttA expression, while miR-981 overexpressing flies exhibit increased CecA1 and AttA expression. Such upregulation of these AMPs was speculated to compensate for the reduced Dpt levels. The fact that in vivo, both miR-9a and miR-981 which act as negative regulators of the IMD pathway are not differentially expressed until their upregulation during the end phase of immune response induction (24 hours post infection), suggests that they maintain immune homeostasis and prevent immune overactivation.

**Regulation of Drosophila Toll pathway by miRNAs**

In Drosophila, miRNAs also regulate the Toll pathway, which is primarily stimulated by fungal and Gram-positive bacterial infections (Figure 2) [61]. Once the pathway is triggered, extracellular protease cascades lead to cleavage of pro-Spatzle to generate the ligand Spatzle which subsequently binds a Toll receptor [62–64]. In the cytoplasm, the adaptor protein MyD88 binds the activated Toll receptor, and recruits another adaptor: Tube, and the kinase Pelle to form the MyD88-Tube-Pelle protein complex [65]. Downstream of this complex is the inhibitor of NF-κB (IkB) factor Cactus and NF-κB transcription factors Dorsal and Dorsal-related immunity factor (Dif), which
in resting conditions are bound to Cactus in the cytoplasm and prevented from transcribing their target genes [66,67]. The formation of the MyD88-Tube-Pelle protein complex results in phosphorylation and degradation of Cactus [68], allowing Dorsal and Dif to translocate to the nucleus and activate transcription of various genes such as AMPs [61,66,67,69,70].

Using a collection of miRNA mutants, a genetic screen identified miRNAs involved in the *Drosophila* response to *C. albicans* infection and Toll signalling. The authors found significantly increased *Drs* expression in miR-277-34 mutants. These flies display increased *Drs* expression after injection with PBS, which is rescued by compromising the Toll pathway component Dif. To separate the effects of *miR-277* from *miR-34*, expression levels of *Drs* were examined in an immune tissue-specific *miR-277* sponge overexpression line. These flies display upregulated *Drs* levels, in line with the *in silico* prediction of *miR-277*’s ability to directly target *Drs* [23]. The opposing effects on *Drs* levels found in *miR-277-34* mutants and *miR-277* sponge overexpressing flies suggest opposite effects of *miR-277* and *miR-34* on Toll signalling and that *miR-34* stimulates innate immune responses as observed for the IMD pathway [24]. However, the role of *miR-34* on Toll signalling remains unsettled as Xiong et al., 2016 found that following Gram-positive *Micrococcus luteus* (*M. luteus*) infection, flies ubiquitously overexpressing *miR-34* exhibit decreased *Drs*, suggesting that *miR-34* downregulates the Toll pathway instead.

The *miR-310-313* cluster is another miRNA cluster that, when knocked out, results in an increased susceptibility to *C. albicans* infection [23]. Interestingly, a separate report found that each member of the *miR-310-313* cluster is capable of negatively regulating Toll signalling through direct targeting of *Drs* [71]. The *in silico* predictions of such targeting were validated using luciferase reporter assay in S2 cells, and *in vivo* using fly lines that either overexpressed or were deficient in a singular member of the miRNA cluster. Overexpression of the entire cluster represses *Drs* more strongly than overexpression of any singular miRNA within the cluster, suggesting that these miRNAs work together synergistically to repress *Drs*. Examination of temporal patterns of *Drs* and miRNA expression in *M. luteus* infected flies revealed that *Drs* peaked at 12 h post-infection and showed upregulation of *miR-310* at 48 h, upregulation of *miR-311* at 24 and 48 h, upregulation of *miR-312* at 24 h, and upregulation of *miR-313* at 12 h post-infection. The upregulation late in the course of infection suggests a role in preventing immune overactivation of this cluster of miRNAs.

The *miR-959-964* cluster, which plays a role in defence against Gram-negative bacteria [59], also possesses roles in Toll signalling. One study found that ubiquitous *in vivo* overexpression of *miR-963* and *miR-964* results in reduced *Drs* expression during *M. luteus* infection [71]. Another report showed decreased survival and decreased *Drs* expression for flies ubiquitously overexpressing *miR-959-962* in response to Gram-positive *Enterococcus faecalis* (*E. faecalis*) infection and increased survival and *Drs* expression in *miR-959-962* knockout flies [72].

An individual member of the *miR-959-964* cluster, *miR-964*, was identified as a negative regulator of Toll pathway signalling. Ubiquitous *in vivo* overexpression of *miR-964* results in increased susceptibility to *E. faecalis* infection and reduced *Drs* expression during Gram-positive *M. luteus* infection, and can be rescued by ubiquitous co-overexpression of a *miR-964* sponge [73,74]. *In silico* prediction of *miR-964* targets and a confirmation study using a luciferase reporter assay in S2 cells showed that *miR-964* directly targets *Drs*. *miR-964* is a miRNA that is differentially expressed during infection, however, unclear patterns of expression following infection make it difficult to discern its specific role. qRT-PCR-based gene expression analysis determined *miR-964* to be upregulated in response to *M. luteus* infection at 24 h, 48 h, and 72 h post-infection [73]. In other reports, at 24 h post-infection, *miR-964-5p* is downregulated while expression of *miR-964-3p* is not differentially changed to statistically significant levels [71,75]. It is possible that circadian cycling of the cluster may impact measurements of expression for miRNAs within the cluster [59].

Individual miRNAs within the *miR-959-962* cluster can also act as negative regulators of Toll
signalling [72]. Ubiquitous in vivo overexpression of each individual miRNAs results in heightened susceptibility to M. luteus infection and decreased Drs expression. In silico prediction and validation using a luciferase reporter assay in S2 cells, and ubiquitous miRNA overexpression in vivo show that miR-959 and miR-960 directly repress tube, miR-961 and miR-962 repress dorsal, and miR-962 represses Toll, all three genes encoding components of the Toll signalling pathway. The dynamic expression of members of this miRNA family was also examined in response to M. luteus infection. Members of the cluster exhibit increased expression only 24 h and 48 h post-infection in the late infection stages. Given the negative regulatory role of these miRNAs, this suggests the role of members of this cluster in re-establishing immune homoeostasis and preventing immune hyperactivation. It was speculated that because miRNAs from the miR-959-962 family are transcribed from the same intron and exhibit similar temporal patterns of expression [59] yet different temporal patterns of repression, the individual miRNAs may have varied half-lives to further fine-tune regulation of Toll signalling. Ubiquitous overexpression of all members of the miR-959-962 family in vivo has a greater effect on Drs repression than overexpression of a singular miRNA of the family, suggesting that these miRNAs work together synergistically to exert their regulatory functions.

miR-317 has also been implicated in negative regulation of Toll signalling during infection [76]. The use of miR-317 ubiquitously overexpressing flies, miR-317 knockout, and a miR-317 and miR-317 sponge co-overexpressing line suggests that miR-317 negatively regulates Drs during M. luteus infection through targeting of the Toll signalling component Dif [66,71,74]. Specifically, miR-317 targets the Dif-RC isoform but not the Dif-RA/RB/RD isoforms, which have different 3’ UTRs and exons [76]. The ability of miR-317 to target the Dif-RC isoform selectively and directly was subsequently confirmed via in silico predictions and a luciferase reporter assay in S2 cells. miR-317 expression impacts the survival of E. faecalis infection with miR-317 ubiquitously overexpressing flies and miR-317 knockout mutants exhibiting decreased and increased survival, respectively. However, reports showing inconsistent patterns of miR-317 expression in wild-type flies make it difficult to evaluate the complete role of miR-317 in immunity. One report employing qRT-PCR found miR-317 to be upregulated at 24 h post-infection with M. luteus [76], which contradicts previous small RNA-seq data demonstrating downregulation of both 3p and 5p species of miR-317 24 h after M. luteus infection [71,75].

miR-958 is another miRNA known to target specific isoforms of components of the Toll signalling pathway [74]. miR-958 ubiquitously overexpressing flies display lower levels of Drs during M. luteus infection and increased susceptibility to E. faecalis infection, while miR-958 null mutants show higher levels of Drs during M. luteus infection [71,74]. In silico predictions suggest that miR-958 targets all Toll and Dif isoforms except Dif-RC, which was verified via a luciferase reporter assay in S2 cells. In vivo, the ubiquitous overexpression of miR-958 revealed that the downregulation of Toll, Dif-RA and Drs expression in these flies could be rescued by the ubiquitous co-overexpression of miR-958 sponge. Given that Dif-RA, Dif-RB, and Dif-RD have identical 3’ UTR sequences, it is likely that miR-958 can repress all of these isoforms. Patterns of expression during infection of miR-958 are unclear, making it difficult to fully discern its role. In wild-type flies infected with M. luteus, the upregulation of miR-958 was shown to occur late during the course of infection at 12 h, 24 h and 48 h post infection [74]. In another study, it was found that at 12 h post infection neither miR-958-3p nor miR-958-5p were differentially regulated and at 24 h, miR-958-3p was downregulated [71,75].

miR-8 is also implicated in negative regulation of innate immune pathways, even in the absence of infection [77,78]. In the absence of septic injury, miR-8 null flies exhibit increased basal levels of Drs and Dpt expression as well as increased frequency of spontaneous melanization [77], a sign of overactive immune pathways [79]. In the absence of infection, the miR-8 null phenotype is detrimental to survival, with just 12% of the flies surviving to adulthood [78]. This phenotype of the miR-8 null mutants could be partially reversed by fat body-specific knockdown of dorsal, as well as ubiquitous knockdown of spatzle and u-shaped
miR-8 (ush) respectively, suggesting a negative regulatory role for miR-8 in Toll signalling and repressing ush to maintain immune homoeostasis and survival. In silico predictions and validation via a luciferase reporter assay in S2 cells showed that miR-8 directly targets Toll and dorsal transcripts [78]. miR-8 represses ush, a known binding partner of GATA transcription factors involved in AMP gene transcription, which also acts as a repressor of Phosphoinositide 3-Kinase (PI3K), a component of the insulin signalling pathway [80–87]. Fly lines overexpressing dorsal and ush in the fat body (the lack of a Toll overexpressing line prevented its testing) showed that these two factors mediate miR-8 regulation of Drs expression [77,78]. However, neither Drs nor Dpt were elevated in two other mutant lines with ubiquitous and fat body-specific inhibition of PI3K [77], suggesting the immunoinhibitory role of miR-8 is not mediated through PI3K. Drs upregulation in miR-8 null larvae is largely dependent on fat body miR-8 levels but not other important immune tissues such as the epidermis and the gut [77]. miR-8 nulls in which miR-8 expression is restored solely in the fat body exhibit rescued Drs levels throughout the body of larvae as well as rescued Drs and Dpt levels in adults and reduced prevalence of melanization.

### Roles of miRNAs in other aspects of Drosophila immunity

In addition to Toll and IMD pathway-mediated regulation of immunity, miRNAs are implicated in regulation of many other genes with roles in innate immunity in Drosophila. miR-956-3p acts as a negative regulator of immunity via repression of Sterile alpha and Armadillo motif (Sarm or Ect4) [88]. Sarm is a Toll/Interleukin-1 Receptor domain-containing protein that contributes to limit IMD pathway activation in Drosophila respiratory epithelial cells during bacterial infection [89]. Mutants carrying a deletion of miR-956, a miRNA whose 3p species is significantly downregulated 3 days post infection with the RNA virus Drosophila C Virus (DCV), exhibit enhanced survival and decreased virus load after DCV infection [88]. Although the deletion of miR-956 removes both the 3p and 5p species, given the low levels of miR-956-5p, such phenotypical changes are likely due to the more abundant miR-956-3p [90,91]. In silico analysis and assays using S2 cells treated with synthetic miR-956-3p mimics show that miR-956-3p represses Sarm [88]. Both wild-type and miR-956 mutant flies upregulate Sarm expression after DCV infection; however, miR-956 mutants induce Sarm expression to a lesser extent than wild-type flies. This suggests that Sarm induction during DCV infection is miR-956 dependent. Further experiments demonstrating decreased survival and increased virus load following DCV infection in Sarm mutants suggest that the decreased miR-956 levels exhibited by wild-type flies in response to DCV infection derepress Sarm and promote innate immunity.

miR-8-5p, which regulates Toll signalling [77,78], also promotes viral resistance through repression of Jun-related antigen (Jra or djun) [92]. In silico prediction and a Spodoptera frugiperda (Sf9) insect cell culture-based reporter assay using the GFP gene cloned upstream of the Jra 3’UTR sequence, confirmed that miR-8-5p represses Jra. In concordance with these results, Jra is upregulated during DCV infection, aligning with the observed in vivo and in vitro downregulation of miR-8-5p during DCV infection [88,92]. In vitro knockdown of miR-8-5p and Jra in S2 cells demonstrates that these two factors promote decreased and increased viral titres, respectively. The role of Jra in promoting viral accumulation and modulating immunity is not yet well characterized, but it has been speculated that Jra may induce host factors involved in viral infection or regulate the apoptosis pathway.

While both miR-8-5p and miR-956-3p are downregulated during DCV infection [88,92], it is not entirely clear whether such decreases are part of the host immune response or caused by the general degradation of RNA during DCV infection [93]. Another miRNA, miR-305-3p, is strongly induced in S2 cells upon infection with the DNA virus invertebrate iridescent virus 6 (IIV6) with a proposed role in virus accumulation [93]. Possible targets of this miRNA include PI3K and the Drosophila homolog of p53 which are often used by viruses in replication [94–97].

miRNAs also play an immune role through maintaining gut barrier function. Loss of function of the miR-34 target dlg1 [24], a mediator of miR-
34 repression of IMD signalling, is implicated in gut barrier dysfunction [98,99]. Such dysfunction from the loss ofdlg1 expression may result in increased leakage of microbes and subsequent upregulation of AMPs [24]. miR-263a also plays an immune–relevant regulatory role in gut function [100]. miR-263a null flies display increased expression of IMD and Toll-related genes including AttA, Dpt, Rel, and Drs as well as increased bacterial loads in the midgut after oral infection with P. aeruginosa. miR-263a mutants also exhibit increased susceptibility to such infection [100], potentially a consequence of the reduced structural integrity of the peritrophic matrix, an insect midgut structure that has previously been shown to play a role in susceptibility to bacterial infection [101].

miR-252-5p works cooperatively with the Forkhead box O (FoxO) transcription factor to modulate innate immunity in the absence of infection through the gene Dawdle (Daw), which encodes a ligand of the TGF-β pathway [102]. The ability of FoxO to target and repress Daw has previously been established [103], and further in silico analysis and confirmation via a luciferase reporter assay in S2R+ cells, an isolate of the S2 cell line [104], show that Daw is a miR-252-5p target [102]. In vivo experiments have addressed the cooperative ability of miR-252-5p and FoxO in downregulating immune activity [102]. Flies with ubiquitous knockdown of either miR-252 or FoxO alone display increased prevalence of melanization in the absence of injury, but the greatest prevalence of melanization is found in flies with ubiquitous knockdown of both miR-252 and FoxO (double mutants). This is further supported by RNA-sequencing and gene expression analysis of these mutant lines. Single-cell sequencing of the double mutants also uncovered increased levels of haemocytes, a class of immune macrophage-like cells [105], further validating the upregulation of immune responses in these flies. It was suggested that miR-252-mediated downregulation of immune activity is mediated through Daw as the ubiquitous knockdown of Daw in addition to miR-252 and FoxO rescues heightened immune activation found in the double mutants [102]. A line with both deletion of Daw’s miR-252 binding site and ubiquitous FoxO knockdown phenocopies miR-252, FoxO double mutants, revealing that miR-252 regulation of immune activity is mediated through Daw rather than other mRNA targets. Downstream of miR-252 and FoxO, Daw modulates immune responses by activating the transcriptional factor Smad on X (Smox) which represses Autophagy-related 8a (Atg8a), a gene in the autophagy pathway [103]. Reduced levels of Atg8a found in miR-252, FoxO double mutants, in combination with knockdown of either Key or Rel which regulate IMD signalling downstream of Atg8a [42], suggest a pathway in which miR-252 and FoxO repress Daw, leading to upregulation of Atg8a activity. Subsequent decreases of Key and Rel activity then downregulate IMD signalling to attenuate inflamming, a term referring to the low-grade chronic inflammation accompanying ageing, which has deleterious effects on lifespan [106]. Supporting this pathway, uninfected flies carrying one mutant copy of either Key or Rel in miR-252, FoxO double homozygous mutant background exhibit increased lifespan, reduced prevalence of melanization, and rescued levels of various Toll and IMD AMPs including Drs, Mtk, and Drosocin [102]. Flies overexpressing Atg8a, miR-252, and FoxO have reduced induction of innate immunity genes and increased lifespan, further validating the ability of these genes to negatively regulate inflamming and innate immunity.

Examination of differentially expressed genes potentially regulated by miRNAs during M. luteus infection also implicates miRNAs in regulating various processes involved in immunity, such as phagocytosis and immune cell proliferation [75], although such regulatory targets of miRNA require further validation.

Conclusions and outlook

In this article, we reviewed the plethora of impacts various miRNAs have on innate immunity as well as the mechanisms by which their expression is regulated. There are still many other miRNAs, which have been predicted to target immunity-related genes [57], been identified as differentially expressed during infection [23,57,71,88] or have been shown to impact immune activity [23,24,57,58,71,107]. However, more research is
needed to confirm such roles and characterize potential mechanisms of regulation.

In the current body of literature, many studies suggest a role for miRNAs in downregulating immune responses, often during the late stages of infection, which results in maintaining immune homoeostasis and avoiding immune hyperactivation. A regulatory network developed through identification of inverse correlations in expression between target genes and potential miRNA regulators at 3 h, 12 h, and 24 h post infection suggests that differential expression of miRNAs at 3 h and 12 h post infection with *M. luteus* promotes a more potent immune response, while at 48 h post infection, just one miRNA of the network is downregulated in comparison to 36 upregulated miRNAs. These many upregulated miRNAs with a wide range of targets involved in immune signalling suggests that the role of miRNAs is in preventing immune overactivation [75]. Examination of these dynamic patterns of expression during infection also reveals that far more miRNAs are differentially expressed at later stages of infection than during earlier stages [23,57,75], causing Wei et al., 2018 to speculate that in the early stages of infection, rapid changes in mRNA may occur to mount a potent immune response, while miRNAs act later to fine-tune such responses [75]. While many miRNAs exhibit such dynamic patterns of expression during infection [57,75], they also impact the basal levels of AMPs [77,78]. The maintenance of immune homoeostasis is crucial, given the well-established trade-offs between immunity and metabolism, reproduction, lifespan, and developmental time [1].

Important areas of future research include the impact of miRNAs on age, time, and sex-dependent differences in immunity. Recently, miRNAs have also been implicated in inflamming [102]. Many miRNAs exhibit differential regulation with age [108–110], circadian time [59], and sex [110]; however, more work is necessary to examine how such differential regulation affects immunity. In addition, most existing studies have examined the role of miRNAs in modulating innate immunity to acute bacterial infection, leaving the role of miRNAs in regulating immune responses to persistent infection and in antiviral immunity as major areas for future research. *mir-956-3p* and *miR-8-5p* have been implicated in antiviral immunity and flies deficient in *Arsenic resistance protein 2 (Ars2)* and the nuclear *cap binding complex (CBC)*, components involved in miRNA silencing were found to be more susceptible to viral infection [88,92,111]. The Toll and IMD pathways also play a role in antiviral immunity [4,5,112], suggesting a possible role for miRNAs in defence against viruses through modulating these pathways. Reciprocally, viruses have also been shown to impact miRNA expression and activity [88,92,113], suggesting a complex interplay between these two elements that requires further study. Evaluation of the dependence of miRNA expression based on pathogen type will also yield insights about which pathways different miRNAs may act in. The use of *Drosophila melanogaster* to study such relationships will prove invaluable in yielding insights regarding miRNA mediated regulation of innate immunity.

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**Data availability**

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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