miR-34 Modulates Innate Immunity and Ecdysone Signaling in Drosophila

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Abstract

MicroRNAs are endogenous small regulatory RNAs that modulate myriad biological processes by repressing target gene expression in a sequence-specific manner. Here we show that the conserved microRNA miR-34 regulates innate immunity and ecdysone signaling in Drosophila. miR-34 over-expression activates antibacterial innate immunity signaling both in cultured cells and in vivo, and flies over-expressing miR-34 display improved survival and pathogen clearance upon Gram-negative bacterial infection; whereas miR-34 knockout animals are defective in antibacterial defense. In particular, miR-34 achieves its immune-stimulatory function, at least in part, by repressing the two novel target genes Dlg1 and Eip75B. In addition, our study reveals a mutual repression between miR-34 expression and ecdysone signaling and identifies genomic elements and transcription factors required for optimal ecdysone-mediated repression of miR-34. Taken together, our study enriches the repertoire of immune-modulating microRNAs in animals, and provides new insights into the interplay between steroid hormone signaling and innate immunity.

Author Summary

MicroRNAs are small regulatory RNAs that impact myriad biological processes. Here we show that the conserved microRNA miR-34 regulates antibacterial defense and steroid hormone signaling in Drosophila. miR-34 over-production or deficiency, respectively, enhances or impairs antibacterial defense. We show that Dlg1 and Eip75B are two novel miR-34 target genes relevant to innate immunity. In addition, our study reveals a mutual repression between miR-34 and steroid hormone signaling and identifies genomic elements and transcription factors required for steroid hormone-mediated repression of miR-34. Thus our study identifies miR-34 as a node linking steroid hormone signaling and immunity, thereby enriching the repertoire of immune-modulating microRNAs in...
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Introduction

Multi-cellular host organisms share the same environment with numerous microbes, and have developed robust defense mechanisms to combat invading microbial pathogens. The fruit fly *Drosophila melanogaster* relies exclusively on innate immunity, the first line of defense, to control microbial infections [1]. For example, upon systemic Gram-negative bacterial infection via septic injury, the immune deficiency (IMD) pathway is activated [2–6], which involves binding of diaminopimelic acid (DAP)-type peptidoglycan (PGN) present in Gram-negative bacteria by the trans-membrane peptidoglycan-recognition protein (PGRP-LCx) and oligomerization of PGRP-LCx. This in turn, leads to the recruitment of the adaptor proteins Immune deficiency (IMD) and Fas associated death domain-containing protein (dFADD), and subsequent recruitment and activation of the Death related ced-3/Nedd2-like caspase Dredd. Next, Dredd cleaves IMD and the neo-C-terminal fragment of IMD binds to and activates the E3 ubiquitin ligase *Drosophila* Inhibitor of Apoptosis protein 2 (DIAP2), leading to poly-ubiquitination of IMD and Dredd, as well as the activation of the MAP3K TGF-β activated kinase 1 (dTAK1) and the *Drosophila* melanogaster IκB kinase complex (DmIKK) [7–13], which phosphorylates the composite *Drosophila* NF-κB protein Relish. In addition, Dredd carries out endoproteolytic cleavage of Relish [14–17]. The N-terminal fragment of Relish translocates to the nucleus and activates the transcription of genes encoding potent anti-bacterial peptides, such as *Diptericin* [18, 19]. In addition, in response to Gram-positive bacterial or fungal infection, the Toll pathway is activated, leading to nuclear translocation of another *Drosophila* NF-κB family member Dorsal-related immunity factor (Dif) and activation of genes encoding potent anti-fungal and anti-bacterial peptides, such as *Drosomycin* [20–24]. Note that some Gram-positive bacteria (e.g. the *Bacillus* species) contain a DAP-type PGN, which is recognized by PGRP-LC [25], whereas PGRP-SD may participate in triggering Toll signaling [26, 27]. Lastly, in addition to the afore-mentioned systemic humoral immunity, which relies on AMPs, *Drosophila* also harbors cellular immunity, which are carried out by specialized hemocytes, including phagocytosis of invading microbes, melanization at the infection sites and encapsulation of larger invading objects such as parasitic eggs [28]. Cellular and humoral immunity work together and constitute a robust defense system that protects *Drosophila* from invading pathogens.

Ecdysone is a steroid hormone essential for *Drosophila* development. Ecdysone binds to the stereotypical steroid hormone receptor complex, a heterodimer composed of the Ecdysone receptor (EcR) protein and its co-factor Ultraspiracle, which functions as a transcription factor and modulates the expression of ecdysone target genes [29]. Ecdysone treatment triggers a rapid activation of a group of early response genes encoding transcription factors (referred to as ecdysone-induced proteins or EIPs), which in turn regulate the expression of late ecdysone response genes. Ecdysone can profoundly alter the gene expression profile both in cultured Schneider (S2) cells and *in vivo*, thereby regulating various key aspects of *Drosophila* development and physiology, including innate immunity [30–32]. For example, it has been reported that ecdysone can activate the expression of PGRP-LC [33], thereby potentiating the IMD innate immunity signaling pathway both in cultured S2 cells and *in vivo*. In addition, ecdysone also strongly regulates the cellular immune response [34, 35].
On the one hand, effective control of pathogens depends on rapid and robust induction of the innate immune response; on the other hand, prolonged or aberrant activation of innate immunity signaling is detrimental to the host, and is associated with a number of pathological conditions in humans. For example, dysregulation of NF-κB signaling contributes to autoimmunity and inflammatory diseases, and can cause several hematopoietic malignancies and various solid tumors [36]. Thus both the magnitude and the duration of innate immunity activation need to be tightly controlled at multiple stages. Genetic screening and gene expression profiling studies have led to the identification of a number of negative modulators of IMD signaling [37]. For example, the amidases peptidoglycan-recognition proteins PGRP-LB and PGRP-SC degrade Gram-negative bacteria peptidoglycan, thereby dampening IMD signaling [38, 39]. In addition, the PGRP-LC-interacting inhibitor of IMD signaling (PIMS)/Pirk/Rudra associates with PGRP-LCx and IMD and causes the depletion of PGRP-LCx from the plasma membrane, thereby suppressing IMD signaling and facilitating to establish immune tolerance to commensal bacteria and maintain a balanced IMD response following oral and systemic infection [40–42]. Furthermore, Caspar, which is homologous to Fas-associating factor 1 in mammals, strongly prevents constitutive activation of IMD signaling by blocking Dredd-dependent nuclear translocation of Relish [43]. Lastly, additional negative regulators of the IMD pathway include the Drosophila homolog of the human cylindromatosis (CYLD) tumor suppressor, a de-ubiquitination enzyme, and the scaffold protein Plenty of SH3 (POSH), which probably operate to inhibit IKK and dTAK1, respectively [44, 45].

In addition to the afore-mentioned protein modulators of innate immunity signaling, non-coding regulatory RNAs such as microRNAs (miRNAs) have also been implicated in immune regulation. miRNAs are a class of 22–24 nt endogenous regulatory small RNAs that function as key regulators of gene expression. miRNAs join the miRNA-induced silencing complexes (miRISC) and guide the miRISC to engage target mRNAs via complementary base-pairing between the seed region of miRNAs (positions 2–8) and miRNA-binding sites (primarily in the 3' UTR of target mRNAs), leading to a reduction in protein output from target mRNAs by a combination of mRNA destabilization and/or translation inhibition [46–52]. A single mRNA can be targeted by multiple miRNAs. Conversely, each miRNA can potentially repress multiple mRNAs. It has been estimated that a significant fraction of mRNAs are subject to miRNA regulation [53].

miRNAs can profoundly impact the intensity and/or duration of immune signaling. For example, in mammals miR-155 is highly induced during the macrophage inflammatory response and contributes to TNFα production [54, 55]. In addition, miR-146a is another mammalian miRNA induced by NF-κB-signaling and controls Toll-like receptor and cytokine signaling by repressing TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1 genes [56]. Similarly, in Drosophila both miR-8 and let-7 have been implicated in fine-tuning IMD and/or Toll signaling [57–59]. However, it is clear that additional miRNAs that confer immune-modulating functions remain to be identified and functionally characterized. We screened a collection of 101 Drosophila miRNAs by examining the impact of their mis-expression in vivo on innate immunity signaling. This led to the identification of miR-34 among several other miRNAs as modulators of IMD signaling. In particular, miR-34 over-expression in cultured cells or in vivo causes aberrant activation of IMD signaling both in the absence and in the presence of immune challenge, and flies over-expressing miR-34 display improved survival and pathogen clearance upon Gram-negative bacterial infection. In contrast, miR-34 mutant flies present profound defects in antibacterial innate immunity. In addition, we found that the immune-modulating role of miR-34 is critically dependent on IMD signaling, and that miR-34 operates in part by repressing genes encoding the septate junction protein Dlg1 and the nuclear hormone family transcription factor Eip75B, a key mediator of the ecdysone steroid.
hormone signaling cascade. Furthermore, our analysis reveals that ecdysone strongly inhibits miR-34 expression via transcriptional repression in a manner that is dependent on a number of transcription factors, including the ecdysone receptor and the Broad Complex (BrC), key mediators of ecdysone signaling cascade. Lastly, we identify ecdysone-responsive regulatory elements required for ecdysone-mediated repression of miR-34 expression. Take together, our study identifies miR-34 as a modulator of innate immunity, identifies both cis-regulatory elements and trans-acting transcription factors required for ecdysone-mediated repression of miR-34, and reveals that the cross-regulation between ecdysone signaling and miR-34 expression contributes to optimal levels of immune activation upon microbial challenge.

Results

Dysregulation of miRNA biogenesis impacts innate immunity signaling

The ribonuclease Drosha is a core component of the miRNA processing machinery essential for the biogenesis of most miRNAs [60]. To examine whether defects in miRNA biogenesis affects innate immunity signaling, we first silenced Drosha expression in vivo by crossing a shRNA transgenic line targeting Drosha (sh-Drosha) to the daughterless-Gal4 (da-Gal4); tub-Gal80ts composite line [61]. A control cross was set up between a shRNA transgenic line targeting gfp and da-Gal4; tub-Gal80ts flies. To minimize lethality due to the requirement for select miRNAs in development, fly crosses were kept at permissive temperature (18˚C). Upon eclosure progeny of appropriate genotype was shifted to restrictive temperature (29˚C) for 5 days to allow for shRNA transgene expression and target gene silencing. This strategy allowed us to achieve significant knockdown of the Drosha mRNA, as measured by RT-qPCR (Fig 1A). Consistent with the critical requirement for Drosha in miRNA biogenesis, depletion of Drosha leads a marked accumulation of several primary miRNA transcripts compared with a control knockdown (tub-Gal80ts da>sh-gfp) (Fig 1A). Importantly, we found that tub-Gal80ts da>sh-Drosha flies display a decrease in AMP expression levels compared with control animals, under both non-infection and E. coli infection conditions (Fig 1B). Interestingly, crosses between da-Gal4 and shRNA-Drosha at room temperature yielded under-represented but viable da>sh-Drosha progeny. As expected, we detected a decrease in levels of the Drosha mRNA and an accumulation of primary miRNA transcripts in these flies (S1 Fig). However, in contrast to the observed decrease in AMP expression in tub-Gal80ts da>sh-Drosha flies, da>sh-Drosha flies display elevated basal levels of AMP expression compared with control animals (S1B Fig). The seemingly disparate phenotype in AMP expression in da>sh-Drosha progeny suggests that the presence and absence of tub-Gal80ts could be attributable to the difference in the onset and duration of Drosha depletion: While Drosha depletion occurred in da>sh-Drosha animals throughout development, it did not take place in tub-Gal80ts da>sh-Drosha flies until fully developed adult animals were shifted to the restricted temperature. For instance, the barrier function of the digestive tract could be compromised in da>sh-Drosha animals due to a requirement for Drosha/miRNAs during development, leading to escape of the microbes and an increase in AMP expression. Nonetheless, these data demonstrate that dysregulation of miRNA biogenesis as a whole impacts the IMD innate immunity signaling pathway.

Over-expression of miR-34 leads to hyperactivation of innate immunity signaling both in cultured cells and in vivo

To further reveal the identities of cellular miRNAs that underlie the innate immunity phenotype, we conducted a miRNA over-expression screen in vivo using 101 UAS-miRNA transgenic lines and the da-Gal4; tub-Gal80ts composite line [62]. A control cross was set up between a
Fig 1. Over-expression of \textit{miR-34} activates innate immunity signaling. (A) Total RNA was isolated from male progeny from crossing flies carrying the ubiquitously expressed \textit{da-Gal4} driver and a temperature-sensitive \textit{Gal80} transgene (\textit{da-Gal4 tub-Gal80\textsuperscript{ts}}) to UAS-shRNA lines targeting Drosha or the control \textit{gfp}. Flies crosses were kept at 18°C and progeny were shifted to 29°C for 5 days upon eclosion to induce shRNA expression. Steady-state levels of mRNAs encoding Drosha and several primary miRNA transcripts were measured by qRT-PCR, and normalized to levels of the \textit{RpL32} mRNA. RNA
isolated from da-gfp shRNA males serves as negative control. (n = 3). (B) Flies were left untreated (+ E. coli) or infected by E. coli via septic injury (+ E. coli), total RNAs were extracted 6 hours post-infection and mRNAs encoding the AMP Diptericin was measured and normalized to levels of Rpl32 (n = 5; mean ± standard deviation (SD)). (C-E) Select miRNAs were over-expressed in flies by crossing UAS-miRNA transgenic lines da-Gal4 tub-Gal80Δ flies. Flies crosses were kept at 18°C and progeny were shifted to 29°C for 5 days upon eclosion to induce miRNA expression. (F) Northern blot shows levels of select miRNAs (right) in control and miRNA over-expression flies. 2S-RNA serves as loading control. Note that levels of the Dipteracin mRNA in non-infected and E. coli-infected da-gfp shRNA flies serve as baseline controls in both D and E (n=4). (F) A Northern blot shows miR-34 expression levels in naïve S2 cells and miR-34 overexpression cells (both were treated with 20-HE at 1 μM for 24 hrs). (G) S2 cells over-expressing miR-34 and control cells were both treated with 20 hydroxy-ecdysone (20-HE) at 1 μM for 24 hrs. Subsequently cells were either left untreated or treated for 6 hrs with a crude lipopolysaccharide sample at 10 μg/mL, which contains the immune stimulator peptidoglycan (PGN). Total RNA was isolated and levels of Dipteracin mRNA were measured by RT-qPCR and normalized to the Rpl32 mRNA (n = 3). (H) Canonical components of IMD signaling were depleted in miR-34 over-expressing cells using dsRNAs targeting IMD pathway components (below) or a control dsRNA against the firefly luciferase gene. Cells were first treated with 20-HE for 24 hours, and subsequently were either left untreated or treated with PGN, and levels of Dipteracin mRNA were measured by RT-qPCR and normalized to the Rpl32 mRNA (n = 3). (I) UAS-miR-34 or the control UAS-sh-gfp flies were crossed to da-Gal4 tub-Gal80Δ flies. Flies crosses were kept at 18°C. Upon eclosion, progeny of appropriate genotypes were collected and shifted to 29°C for 7 days. Flies groups of 45 were subsequently injected with a concentrated culture of Erwinia carotovor a carotovora 15 (Ecc15) or PBS (non-infection control) and kept at 29°C. Fly survival was recorded daily up to 8 post-infection and plotted (n=3; p<0.05 between Ecc15-infected control and miR-34ΔΔ (da-miR-34-Gal80Δ) flies). (J-K) Control or miR-34-TS flies were infected by injecting a concentrated culture of Ecc15 (J) or overnight culture of Enterobacter cloacae (K). At various time points post-infection, groups of 3 flies in J (and groups of 4 flies in K) were collected and homogenized in sterile PBS. Fly homogenates were diluted and plated onto Ampicillin- (in J) or Nalidixic acid-containing (in K) LB plates, and the resultant colonies were counted one day later. Shown are colony-forming units (CFUs) per fly (n=4).

doi:10.1371/journal.ppat.1006034.g001

shRNA transgenic line, which expresses an artificial sh-gfp RNA embedded in the miR-1 cassette [61], and da-Gal4; tub-Gal80Δ flies. Fly crosses were kept at permissive temperature (18°C) until adult progeny of the appropriate genotype emerged. Subsequently the progeny was shifted to restrictive temperature (29°C) for 5 days to allow for miRNA transgene expression. This strategy allowed us to achieve marked over-expression of individual miRNAs. Fig 1C shows results of Northern blot analyses for select miRNAs. Under these experimental conditions, viable adult progeny of the appropriate genotype that over-expresses individual miRNAs was readily recovered. One exception was let-7, which causes lethality at pupal stage (S1 Table).

Subsequently, these flies were either left untreated or infected with E. coli via septic injury. Total RNA was isolated 6 hours post-infection and levels of the Dipteracin (Dpt) mRNA were measured and normalized to the Rpl32 control mRNA. S1 Table documents the relative Dipteracin mRNA levels in uninfected and E. coli-infected flies compared to the respective controls (da>sh-gfp flies that are uninfected and infected by E. coli). The AMP expression phenotype for select miRNAs (miR-34, miR-92a, miR-9a and miR-989) is shown (Fig 1D and 1E). In subsequent studies, we focused on miR-34 because 1) miR-34 over-expression causes a concordant increase in levels the Dipteracin mRNA in both uninfected and E. coli-infected flies; and 2) miR-34 expression is regulated by ecdysone signaling, which impacts innate immunity [33, 63].

Besides IMD signaling, which is activated upon Gram-negative bacterial infection, we also asked whether miR-34 additionally impacts other signaling routes, such as the Toll signaling pathway, which mediates host defense against infection by fungi and Gram-positive bacteria. Interestingly, our analysis revealed that Drosomycin expression in response to M. luteus infection was significantly decreased in miR-34 over-expressing flies compared with controls (S2 Fig), suggesting that miR-34 differentially impacts IMD and Toll signaling. Next, we examined the impact of miR-34 over-expression on AMP expression in immuno-competent cultured S2 cells. We detected higher levels of the Dipteracin mRNA, as well as a panel of mRNAs encoding additional AMPs, including Cecropin A1 (Ceca1), Attacin A (AttA), Metchnikowin (Mtk) and Defensin (Def), in miR-34 overexpressing cells than in control cells, both in the absence and
presence of peptidoglycan (PGN) treatment (Fig 1F and 1G, S3 Fig). In addition, levels of the pirk mRNA, which is transcriptionally activated by IMD signaling and encodes a negative regulator of IMD signaling, were also increased both in untreated and PGN-treated cells upon miR-34 over-expression (S4 Fig). Importantly, depletion of core components of the IMD signaling pathway, such as PGRP-LC, subunits of the DmIKK complex (Ird5 and Kenny), or Relish, significantly alleviated the immune-activation phenotype of miR-34 (Fig 1H), indicating that the canonical IMD signaling pathway is required for the immune-stimulating function of miR-34. Interestingly, in ecdysone-treated S2 cells the effect of miR-34 overexpression appears to be PGRP-LC-dependent, even in the absence of PGN stimulation. It is possible that miR-34 overexpression may impact ecdysone-mediated regulation of PGRP-LC expression [33], thereby affecting IMD signaling. Taken together, these data demonstrate that mis-expression of miR-34 affects the IMD innate immunity signaling pathway both in cultured cells and in vivo.

**miR-34 impacts innate immunity signaling in vivo**

To further define the role of miR-34 in innate immunity signaling in vivo, we injected either sterile PBS or a suspension of a concentrated culture of the fly pathogen Erwinia carotovora carotovora strain 15 (Ecc15) into miR-34 over-expression or control flies, and monitored fly survival at different time points post injection. As expected, flies of both genotypes survive well in response to PBS injection (Fig 1I). Importantly, while flies of both genotypes show a decrease in survival following Ecc15 infection, miR-34 over-expression flies display a significantly higher survival rate than control flies (Fig 1I), consistent with the observed higher levels of AMP expression in miR-34 over-expression flies. To further examine whether enhanced survival of miR-34 over-expression flies upon Ecc15 infection is attributable to improved pathogen clearance or immune tolerance, we monitored pathogen load at various time points post Ecc15 infection. Our analysis revealed that while infected flies of both genotypes carry comparable amount of Ecc15 at the starting time point, miR-34 over-expression flies appear to outperform control animals in clearing invading pathogens, as indicated by the lower pathogen load in miR-34 over-expression flies than in controls (Fig 1J). Similar observations were made upon infecting flies with, Enterobacter cloacae, a second Gram-negative bacteria (Fig 1K). Furthermore, to examine whether miR-34 over-expression affects hemocyte-mediated bacterial phagocytosis, which is a key mechanism of the cellular immune response, we injected into flies E. coli bio-particles conjugated with a pH-sensitive dye, which becomes fluorescent only after being engulfed by hemocytes and sorted into the acidic endosomal compartment. This analysis reveals a similar degree of phagocytosis between control and miR-34 over-expression flies (S5A and S5B Fig). We conclude that miR-34 over-expression enhances innate immunity and host survival upon Gram-negative bacterial infection, at least in part, by promoting AMP expression and pathogen clearance.

Next, we examined whether miR-34 deficiency impacts innate immunity by measuring AMP expression both in miR-34 knockout (miR-34KO) flies [64] and in control (Res) animals (miR-34KO flies carrying a miR-34 genomic rescue construct) (Fig 2A). This analysis revealed that miR-34KO flies express significantly lower levels of the Dipterycin mRNA than control animals, under both non-infection and E. coli infection conditions (Fig 2B). To alleviate the concern that endogenous or environmental microbes could potentially impact host innate immunity and cause variations in levels of AMP expression, we also analyzed miR-34KO and wildtype flies raised in media containing multiple antibiotics. We note that antibiotics treatment alone may not completely eliminate microbes. Nonetheless, under these conditions, lower levels of Dipterycin expression was detected in miR-34KO animals than in control animals
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**Fig 2.** miR-34 deficiency compromises innate immunity. (A) A Northern blot shows levels of miR-34 and the control 2S rRNA in miR-34 knockout flies (KO) or knockout flies carrying a miR-34 rescue transgene (control, ctr). (B) Files were either uninfected or infected with *E. coli* via septic injury. Total RNA was isolated and levels of the Dipterin mRNA were measured by RT-qPCR and normalized to the RpL32 control (mean ± SD; n = 3). (C-D) miR-34KO and control flies were reared in standard food supplemented with antibiotics. Age-matched fly progeny (young– 3d, old– 22d) were either uninfected (C) or infected with *E. coli* via septic injury (D), and levels of the Dipterin mRNA were measured by RT-qPCR and normalized to the RpL32 control (n ≥ 3). In both panels, the Dpt/RpL32 ratio in non-infected 3d old control flies serves as baseline. (E-F) Groups of 45 age-matched miR-34KO and control flies (E, young– 3d; F, old– 23d) were injected with a concentrated culture of Ecc15 or PBS. Fly survival was recorded daily and plotted (n ≥ 3; p < 0.001 between Ecc15-infected control and miR-34KO flies). (G-H) A similar group of age-matched control or miR-34KO flies (as in E and F) were infected by injecting a concentrated culture of *Enterobacter cloacae*. At various time points post-infection, groups of 4 flies were collected and homogenized in sterile PBS. Note that due to lethality, 1 fly per group was used for a subset of data points in miR-34KO flies 2 days post-infection. Fly homogenates were diluted and plated onto Ampicillin-containing LB plates, and the resulting colonies were counted one day later. Shown are colony-forming units (CFUs) per fly (n ≥ 5). (I) Groups of 4–7 d miR-34KO and control flies were injected with a concentrated culture of *Enterobacter cloacae* or PBS. Fly survival was recorded daily and plotted (n ≥ 3; p < 0.001 between *Enterobacter cloacae*-infected control and miR-34KO flies). (J) A similar set of flies (as in I) were infected by injecting an overnight culture of *Enterobacter cloacae*. At various time points post-infection, groups of 4 flies were collected and homogenized in sterile PBS. Fly homogenates were diluted and plated onto Nalidixic acid-containing LB plates, and the resulting colonies were counted one day later. Shown are colony-forming units (CFUs) per fly (n ≥ 5).
(Fig 2C and 2D). Consistent with lower levels of Dpt expression, miR-34KO flies present poorer survival and pathogen clearance in response to Ecc15 compared to control animals (Fig 2E). Lastly, since miR-34 expression steadily increases with age, we also examined AMP expression, host survival and pathogen clearance upon Ecc15 infection in various groups of age-matched miR-34KO and control flies. Consistent with previous reports [65–67], we detected higher basal levels of Diptericin transcript in aged control flies than in young flies (Fig 2C). Notably, this age-dependent increase in basal levels of AMP expression persists in miR-34KO animals, suggesting that age-dependent increase in miR-34 expression does not significantly contribute to the observed difference in AMP expression between young and old flies. Importantly, in both young and aging settings, miR-34KO flies display lower basal and E. coli infection-induced levels of Diptericin expression, poorer survival and pathogen clearance in response to Ecc15 challenge compared to age-matched control animals (Fig 2C–2H). Lastly, our analysis reveals that miR-34KO flies display a defective survival and pathogen clearance in response to Enterobacter cloacae infection (Fig 2I and 2J), and that miR-34 deficiency did not significantly impact hemocyte-mediated phagocytosis (S5C and S5D Fig). These data demonstrate that miR-34 deficiency compromises AMP expression and impairs IMD signaling.

Identification of miR-34 target genes relevant to innate immunity signaling

To identify miR-34 targets, we first employed two widely used bioinformatics algorithms (TargetScan and PicTar) [46, 68, 69]. In addition, we performed mRNA sequencing and compared the mRNA expression profiles in cells over-expressing miR-34 with that in control samples. As most miRNAs only mildly reduce the expression of cognate target genes, we undertook an inclusive approach and considered all mRNAs that display a decrease in expression upon miR-34 over-expression. Integration of these datasets identified a list of 27 genes that not only scored positively using both bioinformatics algorithms, but also displayed a decrease in mRNA levels upon miR-34 over-expression (S6 Fig & S2 Table). Among these 27 genes is Eip74EF, which is a well-characterized miR-34 target, thereby validating our approach [64]. Next, to examine whether any of these 27 genes are functionally relevant to innate immunity signaling, we knocked down these genes individually in S2 cells and examined the impact on Diptericin expression prior to and after PGN treatment. This analysis revealed that inactivation of three genes, CG8468, dlg1 and mura, led to an increase both in basal levels and/or PGN-induced Diptericin expression (Fig 3A). While the magnitude of changes in Diptericin expression upon silencing of individual genes appears to be weaker than that elicited by miR-34 overexpression, possibly due to low knockdown efficiency and/or slow turnover of target proteins, these data nonetheless suggest that CG8468, dlg1 and mura are candidate miR-34 target genes that could potentially downregulate IMD signaling.

Validating dlg1 and Eip75B as bona fide miR-34 target genes that modulate innate immunity signaling

Dlg1 is a member of membrane-associated guanylate kinase (MAGUK) family proteins (albeit it lacks catalytic activity), and is localized to septate junctions [70]. Depletion of Dlg1 in cultured S2 cells led to an increase in both basal levels and/or PGN-induced Diptericin expression (Fig 3A and 3B). In addition, we assessed the impact of Dlg1 depletion on innate immunity signaling in flies using UAS-shRNA transgenic lines (sh-dlg1 and the sh-gfp control) and the da-Gal4; tub-Gal80ts composite line. As expected, levels of the dlg1 mRNA are reduced in da>sh-dlg1 flies compared with that detected in the control da>sh-gfp flies (Fig 3C). Importantly, such a decrease in dlg1 mRNA levels correlated with an increase in levels of the Diptericin mRNA
Fig 3. *dlg1* is a *miR-34* target gene relevant to innate immunity signaling. (A) S2 cells treated with various dsRNAs (below) were first treated with 20-HE for 24 hrs, and were subsequently either left untreated or treated with PGN. Total RNA was isolated and levels of *Diptericin* were measured and normalized to *RpL32* (n = 3). *Imd* dsRNA serves as a positive control. (B) Levels of Dlg1 protein in *dlg1* knockdown cells or control cells were measured by immunoblot. Tubulin serves as a loading control. The remaining Dlg1 protein reflects incomplete depletion. (C-D) *Dlg1* was knocked down in flies using a UAS-shRNA and the da-Gal4 tub-Gal80*ts* transgenes in a similar strategy as described in Fig 1. Levels of *dlg1* (C) and *Diptericin* mRNA (D) in *E. coli*-
infected flies were measured by qRT-PCR, and normalized to Rpl32. RNA isolated from progeny of a parallel cross using the sh-gfp transgene serves as negative control (n = 3). (E-F) Levels of dlg1 mRNA (E) or protein (F) in S2 cells over-expressing miR-34 or in control cells were measured. The Rpl32 mRNA and Tubulin protein serve as controls, respectively (n = 3). The remaining Dlg1 protein in F reflects incomplete depletion. (G-H) Levels of Dlg1 protein in miR-34 knockout or control flies were measured by immunoblot (G). Note that multiple Dlg1 protein isoforms were detected in fly extracts. Their collective abundance was quantified and shown in H (n = 3; mean + SD). (I) Renilla luciferase reporter constructs that carry the dlg1 3' UTR containing either a wildtype (WT) or mutant (mut) miR-34 binding site were generated. Seed region of miR-34 was highlighted in green. (J) The Renilla luciferase reporter constructs (in J) together with a control firefly luciferase construct were transfected into S2 cells with or without a miR-34 expression vector. Reporter activities were measured and relative Renilla/Firefly ratio is shown (n = 3). (K) Various combinations of miR-34 and Flag-Dlg1 expression constructs were transfected into S2 cells. Total RNA was isolated and levels of the Dipteracin mRNA were measured and normalized to Rpl32 (upper panel; n = 3). Levels of Dlg1 protein were measured by immunoblot (lower panel).

doi:10.1371/journal.ppat.1006034.g003

(Fig 3D). These data demonstrate that a reduction in levels of Dlg1 correlates with enhanced IMD signaling both in cultured cells and in vivo. Consistent with the notion that dlg1 is a miR-34 target gene, we observed a reduction in both mRNA and protein levels of dlg1 upon over-expression of miR-34 (Fig 3E and 3F). In addition, higher levels of Dlg1 protein were detected in miR-34 knockout flies than in control animals (Fig 3G and 3H). Furthermore, our bioinformatics analysis predicted a miR-34 binding site in the dlg1 3' untranslated region (3' UTR) (Fig 3I). To show definitively that the predicted miR-34 binding site is sufficient to confer gene silencing, we performed reporter assays using luciferase reporter constructs carrying either the wildtype or mutant dlg1 3' UTR, which abolishes miR-34 binding (Fig 3I). This analysis reveals that miR-34 is capable of silencing the reporter construct carrying a wildtype dlg1 3' UTR, and this repression is relieved by introducing mutations in the miR-34 binding site (Fig 3I). Lastly, we found that while miR-34 over-expression led to a significant reduction in levels of Dlg1 protein, expression of a dlg1 cDNA construct containing only the open reading frame efficiently restored Dlg1 protein levels in S2 cells even in the presence of exogenous miR-34 (Fig 3K, lower panel). This is consistent with the notion that a functional miR-34 target site is present only in the 3' UTR of the Dlg1 mRNA. Importantly, expression of dlg1 efficiently blunted the immune-stimulating effect of miR-34 (Fig 3K, upper panel). Taken together, these data demonstrate that dlg1 is a bona fide (perhaps a major) miR-34 target gene relevant to IMD signaling.

Among our list of miR-34 targets is Eip74EF, which encodes a key component of the ecdysone signaling cascade and has been previously identified as a miR-34 target gene [64]. We confirmed these findings by showing that over-expression of miR-34 can lead to a significant reduction in the levels of Eip74EF protein (Fig 4A and 4B). Inspired by the notion that a single miRNA can coordinately regulate the expression of multiple components of a given pathway [71], we performed immunoblot assay and surveyed additional ecdysone response genes to search for additional miR-34 targets. This analysis revealed that protein levels of Eip75B, a transcriptional factor and component of ecdysone signaling, are significantly reduced by miR-34 over-expression (Fig 4A and 4B). Further inspection of the Eip75B mRNA sequence allowed us to identify five potential miR-34 sites in the coding region. In order to test whether any of these sites can confer miR-34-mediated gene silencing, we generated reporter constructs by placing tandem triple repeats of individual miR-34 binding sites derived from the Eip75B mRNA in the 3' UTR of the firefly luciferase gene, and examined whether these reporter genes can be repressed by miR-34. In addition, we generated a corresponding set of reporter constructs carrying mutations in candidate miR-34 binding sites that abolish miR-34 recognition. This analysis reveals that reporter genes containing wild type sites #1 or #5 were more efficiently repressed by miR-34 than the reporter with mutant sites (Fig 4C and 4D & S7 Fig). In addition, depletion of Eip75B in S2 cells resembles the miR-34 over-expression phenotype, i.e. activation of innate immunity signaling both in the presence and absence of PGN treatment (Fig 4E and 4F). These observations are consistent with previous studies that identified Eip75B
as a negative regulator of IMD signaling [33, 72]. Importantly, over-expression of the Eip75B-RC isoform, which lacks site #1, significantly suppressed the immune-activation function of miR-34 (Fig 4G). We conclude that besides dlg1, Eip75B is another miR-34 target gene relevant to innate immunity signaling.
miR-34 is transcriptionally repressed by ecdysone signaling

It has been reported that 20-hydroxyecdysone (20-HE) treatment in S2 cells can profoundly alter the expression levels of a number of miRNAs. For example, expression of let-7, miR-100 and miR-125, which cluster at the same genomic locus, is markedly induced upon 20-HE treatment [63]. In contrast, levels of miR-34 display a significant decrease in response to 20-HE treatment. We confirmed the inhibitory effect of 20-HE on miR-34 expression by performing Northern blot to quantify levels of mature miR-34 in S2 cells before and after 20-HE treatment (Fig 5A and 5B). Such ecdysone-mediated modulation of miR-34 gene expression is most likely at the level of transcription, as changes in levels of the primary miRNA transcripts resemble that of mature miRNAs (Fig 5A–5C) [63]. Further supporting this notion, a miR-34 transgene driven by the metallothionein promoter was robustly expressed in the presence of 20-HE, while its endogenous counterpart under the control of cognate regulatory elements was strongly repressed under the same conditions (Fig 1F).

Ecdysone receptor (EcR) and the Broad complex (BrC) are essential for ecdysone signaling. EcR heterodimerizes with its co-factor Ultraspiracle, and functions as a stereotypical steroid hormone receptor to modulate the expression of ecdysone target genes, whereas the transcription factor BrC is an ecdysone-induced early gene product that regulates the expression of...
downstream ecdysone-responsive genes [29, 73, 74]. Consistent with previous reports showing that let-7 expression is activated by ecdysone signaling, depletion of ecdysone EcR or BrC in S2 impaired the increase in levels of the pri-let-7 transcript after 20-HE treatment (Fig 5D and 5E & S8 Fig) [59, 63]. In addition, while BrC has been implicated in ecdysone-mediated repression of miR-34, the requirement for BrC in this process could not be definitively assessed [63]. We therefore measured changes in levels of both the primary miR-34 transcript and mature miR-34 elicited by BrC or EcR knockdown. This analysis revealed that the repressive effect of ecdysone on miR-34 expression was partially relieved in EcR or BrC knockdown cells compared with control RNAi cells (Fig 5A–5F). Furthermore, consistent with the findings of Silverman, Ambros and colleagues [33, 63], we detected higher levels of pri-miR-34 in BrC mutant larvae than in control animals, and found that the BrC mutant larvae expressed lower levels of Dpt in response to E. coli infection (Fig 5G and 5H). Taken together, these data demonstrate that both EcR and BrC are required for optimal ecdysone-mediated transcriptional repression of miR-34.

We noticed that while BrC dsRNA treatment led to a significant reduction in both mRNA and protein levels of BrC, 20-HE-mediated repression of miR-34 was only partially relieved (Fig 5A–5F). These observations suggest that there are yet-to-be-identified factors that are required for 20-HE-mediated miR-34 repression. A recent study reported that the consensus binding motif for a collection of transcriptional factors, including Pnr, Aef1, Trl, CrebA, Srp, Twi and Ap, are enriched in candidate regulatory regions that confer ecdysone-mediated transcriptional repression [75]. We therefore examined whether these transcription factors contribute to ecdysone-mediated repression of miR-34. In addition, we also included in our test additional components of the ecdysone signaling pathway, such as Hr46, E78C and E93F. Our analysis revealed that three additional transcription factors (Srp, Twi and Ap) are required for optimal repression of miR-34 by ecdysone (Fig 5I, S9 Fig).

Identification of cis-regulatory elements responsible for ecdysone-mediated repression of miR-34

The miR-34 genomic locus contains two additional miRNA genes, miR-277 and miR-317, as well as a protein-coding gene Fmr1 (Fig 6A). While all the three miRNA genes are repressed by 20-HE treatment, little changes in levels of the Fmr1 mRNA were detected (Fig 6B and 6C). As a control, 20-HE treatment led to a marked increase in levels of the BrC and Eip75B mRNA (Fig 6B). These observations suggest that local regulatory sequences may confer 20-HE-mediated repression of these miRNA genes. Recently the Stark group has employed STARR-seq (self-transcribing active regulatory region sequencing) to identify enhancers at a genome-scale [76]. STARR-seq exploits the ability of enhancers to function independently of their relative positions. If candidate enhancers are placed downstream of a minimal promoter, such that active enhancers transcribe themselves, each enhancer’s strength will be reflected by its abundance among cellular RNAs. This approach has identified several genomic regions near the miR-34 locus (referred to as ecdysone-responsive peaks P1 through P5) as putative ecdysone-responsive elements (Fig 6A) [75]. To examine whether these DNA elements can mediate ecdysone-dependent repression, we generated reporter constructs by placing individual genomic fragments upstream of a Drosophila Synthetic Core Promoter (DSCP) that drives the firefly luciferase reporter gene. Reporter assays reveal that each of the five regions can confer ecdysone-mediated repression with varying strengths. Notably, among the five genomic fragments tested, peak P2 displays the most robust repressive effect in response to ecdysone treatment (Fig 6D). Of note, all three ecdysone-repressed miRNA genes are transcribed in the same direction, whereas the ecdysone-insensitive Fmr1 gene is transcribed in the opposite direction.
to the miRNA genes. Consistent with the notion that peak P2 is the major regulatory element that confers sensitivity to ecdysone signaling, it is physically located within a closer distance to the miRNA genes than to the \textit{Fmr1} gene (Fig 6A).

Since BrC is required for optimal ecdysone-mediated repression of \textit{miR-34}, we inspected the nucleotide sequence of peak P2 and identified five putative BrC-binding sites (shown as shaded boxes in Fig 6E). To validate and assess BrC occupancy on these regions \textit{in vivo}, we...
performed chromatin immunoprecipitation (ChIP) assay both in naïve S2 cells and cells treated with 20-HE, using an antibody against BrC. As expected, in the absence of ecdysone treatment, BrC is expressed at very low levels and thus no enrichment can be detected. However, upon 20-HE treatment, we detected various degree of BrC enrichment in these regions, with F3 displaying the most significant BrC occupancy compared with a control ChIP using a non-immune (n.i.) serum (Fig 6F).

To further define the minimal cis-regulatory element within peak P2 that are required for miR-34 repression, we placed various truncated P2 genomic fragment upstream of the reporter gene and assessed their capability of conferring ecdysone-mediated silencing. This analysis revealed that any deletions that perturb the region composed of nucleotides 573–587 compromised the repressive effect of ecdysone (Fig 6E and 6G). Notably, the genomic fragment F3, which displays the highest degree of BrC occupancy, centers around nucleotides 573–587 (Fig 6E and 6F), suggesting that BrC binding at this region is required, at least in part, for the repressive effect of ecdysone on miR-34 expression. Taken together, these analyses identified key cis-regulatory genomic elements and trans-acting transcription factors required for ecdysone-mediated repression of miR-34.

Discussion

In this study, we identify the microRNA miR-34 as a link in the intricate interplay between ecdysone signaling and innate immunity. We show that over-expression of miR-34 either in flies or in cultured S2 cells leads to hyper-activation of antimicrobial peptide gene expression both in the absence and in the presence of immune challenge, and enhances pathogen clearance in vivo, and that miR-34 deficiency compromises innate immunity. In addition, miR-34 modulates IMD signaling, in part, by repressing genes encoding the septate junction protein Dlg1 as well as Eip75B, a component of the ecdysone signaling cascade and a negative regulator of the IMD pathway. Furthermore, our analyses reveal that miR-34 is transcriptionally repressed by the ecdysone signaling cascade in a manner that is dependent on the ecdysone receptor and the transcription factor BrC. Moreover, we characterize hormone-responsive cis-regulatory regions in the miR-34 locus and trans-acting transcription factors required for ecdysone-mediated repression of miR-34. Lastly, we show that miR-34 represses the expression of a number of components in ecdysone signaling, including Eip74EF, Eip75B and BrC. Thus our study uncovers miR-34 as a component of an ecdysone-dependent regulatory circuit that modulates IMD innate immunity signaling in Drosophila.

Ecdysone-mediated activation of the EIP genes is achieved mainly by robust transcriptional activation, as mRNAs encoding EIP proteins accumulate rapidly upon ecdysone treatment, followed by a massive increase in levels of the EIP proteins. Eip74EF, a validated miR-34 target gene, is among such group of early response genes. In flies lacking miR-34, dys-regulated expression of Eip74EF is linked to accelerated aging in the brain and shortened lifespan [64]. Here we identify Eip75B, another early response gene, as a new target of miR-34. In addition, levels of the BrC protein, which is a transcriptional factor and component of ecdysone signaling, is also significantly reduced upon miR-34 over-expression (Fig 4A and 4B). Of note, the BrC transcript harbors a predicted miR-34 binding site, which could underlie miR-34-mediated repression [46, 68]. Thus, our study lends strong support to the notion that certain miRNAs can coordinately target multiple components of a given biological process to achieve effective regulation by adding miR-34 to such collection of miRNAs [71]. In addition, considering that the miR-34 gene itself is transcriptionally repressed by ecdysone signaling, our findings indicate that ecdysone signaling not only activates select EIP genes transcriptionally to boost levels of the corresponding mRNAs, but also maximize the protein output from these
mRNAs by reducing levels of miR-34. Thus, our study suggests that the cross-regulation between ecdysone signaling and miR-34 expression appears to constitute a positive feedback loop, which may facilitate to achieve appropriate levels of output from ecdysone signaling under various physiological conditions. For example, several ecdysone bursts occur during early stages of Drosophila development [77]. As a consequence, ecdysone signaling prevails and keeps miR-34 levels low. This ensures optimal output from ecdysone signaling, which is crucial for many aspects of development. In fact, flies lacking miR-34 develop normally, consistent with the observed low levels of miR-34 expression during early stages of development [64]. In contrast, levels of miR-34 increase drastically in newly hatched adults and display a further elevation with age [64]. Coincidently ecdysone levels decline during pupae-adult transition and remain low in adult flies [77]. Thus it appears that in adult flies, miR-34 expression prevails and further represses components of ecdysone signaling. Such repression of ecdysone signaling is physiologically relevant to adult flies. In fact, miR-34 knockout flies display an early onset of neuro-degeneration during aging compared with age-matched wildtype counterparts. This phenotype is at least in part, attributable to elevated levels of Eip74EF, which on the one hand plays a key role in ecdysone signaling during early stages of fly development, and on the other hand, seems to become detrimental in adults [64].

While it is clear that ecdysone treatment primes S2 cells to become competent in innate immunity signaling, it appears that the underlying molecular mechanism is rather complex (Fig 7). For example, ecdysone signaling strongly activates the expression of Eip74EF and Eip75B, encoding transcription factors that operate as an activator and repressor of innate immunity signaling, respectively [33, 72]. In addition, with respect to miRNAs, ecdysone treatment markedly activates and inhibits, respectively, the expression of the miRNA genes let-7 and miR-34 [63]. Considering a previous report showing that let-7 represses Diptericin [59] and the pro-immunity role of miR-34 uncovered in this study, it would have been expected...
that ecdysone would reduce the output of the IMD innate immunity signaling pathway. Furthermore, our findings showing that miR-34 can repress multiple genes of ecdysone signaling, including Eip74EF, Eip75B and BrC, add an additional layer of complexity. It is possible that these events take place at different phases of innate immunity signaling (e.g. initiation, maintenance and post-induction repression), reminiscent of the regulation of Pirk, which is activated at early phase of IMD signaling and represses the IMD pathway at a later phase [40–42]. Lastly, the newly identified miR-34 target genes further expand the repertoire of potential negative regulators of IMD signaling. These genes display discrete expression profiles during development and immune activation, as they are likely subject to additional regulatory mechanisms besides miR-34. At any given time point, the output of innate immunity and ecdysone signaling is the net sum of outcomes from multiple regulatory processes. This may facilitate to achieve optimal outputs of innate immunity and ecdysone signaling during development and/or upon immune challenge. The precise sequence and interaction of events during the intricate interplay between ecdysone signaling and innate immunity during development, as well as the underlying mechanism remain to be determined.

Beside Eip75B, our study also identifies dlg1 as another miR-34 target gene relevant to innate immunity signaling. We show that 1) Dlg1 depletion resembles the phenotype elicited by miR-34 over-expression; 2) miR-34 over-expression causes a marked reduction in both mRNA and protein levels of Dlg1; 3) the 3’ UTR of the dlg1 mRNA contains a functional miR-34-responsive site; and 4) Over-expression of dlg1 abrogates the immune-stimulatory activity of miR-34. Dlg1 is localized to septate junctions and is a member of membrane-associated guanylate kinase (MAGUK) family proteins. Our findings are consistent with a recent study showing that the big bang (bbg) gene, which encodes a PDZ domain-containing protein that is present at the septate junctions, is required for gut epithelial barrier integrity. Flies homozygous for mutations in the bbg gene display constitutive activation of AMP genes in the gut by resident gut microbes [78]. Given that loss of dlg1 in flies correlates with gut epithelial barrier dysfunction [79], and that knockdown of dlg1 in mammals can attenuate the gut barrier integrity [80], an attractive possibility is that loss of dlg1 in flies could lead to gut epithelial barrier dysfunction and constitutive expression of AMP genes due to leakage of gut microbes. In addition, our observations that Dlg1 also modulates the IMD pathway in cultured S2 cells also suggest a signaling role of Dlg1. For example, the mammalian scaffold protein CARMA1/CARD11, a member of the MAGUK family proteins, has been implicated in NF-κB signaling. In particular, CARD11 forms a complex with the adaptor protein BCL10 and the paracaspase MALT1 and mediates the activation of the NF-κB pathway upon T-cell activation [81, 82]. However, our attempt to uncover interactions between Dlg1 and canonical components of the IMD signaling pathway, including IMD and Kenny, was unsuccessful. Of note, since the miR-34 effect on immunity is PGRP-LC-dependent, it would be interesting to assess potential interactions between Dlg1 and PGRP-LC. The molecular mechanism by which Dlg1 modulates innate immunity signaling warrants further investigation.

We noticed that levels of the Dlg1 protein display a steady decrease in S2 cells upon PGN treatment (S10 Fig). In addition, consistent with the observation made in S2 cells, we also detected a gradual decrease in levels of the Dlg1 protein over time in E. coli-infected flies (S11A Fig). Of note, levels of miR-34 remain unchanged in E. coli-infected flies (S11B and S11C Fig), indicating that the observed decrease in Dlg1 protein levels is attributable to a miR-34-independent mechanism. Moreover, we detected a general reduction in dlg1 mRNA levels upon depletion of canonical components of the IMD signaling pathway in S2 cells (S12 Fig). Lastly, a moderate drop in levels of the dlg1 mRNA was detected at early stages of ecdysone treatment in S2 cells (S13 Fig). Taken together, these analyses suggest that the regulation of dlg1 gene expression is mediated by both miR-34-dependent and miR-34-independent mechanisms.
We found that RNAi-mediated silencing of Su(Z)12, another predicted miR-34 target gene [83], led to an elevation of Diptericin mRNA levels (S14 Fig). Thus, it appears that miR-34 modulates innate immunity signaling by repressing multiple target genes. Our study joins other studies to support the notion that the function of a given miRNA is generally dictated not by a single, but rather by a cohort of target genes, and that the net biological outcome is reflected as a collective effect of multiple target genes.

Expression of miR-34 is subject to complex regulation during the fly life cycle. miR-34 is expressed at relatively low levels during early stages of development and is strongly repressed by ecdysone signaling at larval and pupal stages. Strikingly, miR-34 levels are significantly elevated in adult flies upon eclosion and with age [64]. However, activation of IMD signaling does not appear to strongly affect miR-34 expression, as levels of miR-34 remain essentially unchanged in the body of flies upon E. coli infection (S11B and S11C Fig). Considering previous reports showing that innate immunity signaling is activated under various stress conditions and in aged flies, and that levels of Diptericin display an increase during aging [65–67], it is possible that elevated miR-34 expression in aged flies may contribute to an increase in stress, which in turn leads to activation of innate immunity signaling pathways. Alternatively, miR-34 may effect IMD signaling by repressing the afore-mentioned cohort of target genes, including dlg1, Eip75B and Su(Z)12, which encode negative regulators of IMD signaling. These two scenarios are not mutually exclusive.

Lastly, our study reveals that ecdysone signaling represses miR-34 by transcriptional inhibition. We identify a cis-regulatory region (encompassing F3, Fig 6E) from the miR-34 locus required for optimal ecdysone-mediated repression of miR-34. Interestingly, the identified genomic element coincides with the region that displays a high degree of occupancy by BrC, an ecdysone-induced transcription factor required for the optimal repression of miR-34 by ecdysone. These observations suggest that BrC may directly contribute to ecdysone-mediated repression of miR-34 by binding to the F3 region. Of note, three additional transcription factors, including Srp, Twi and Ap, also contribute to ecdysone-mediated repression of miR-34, and predicted Srp- and Twi-binding sites can be found within and close to F3, respectively (Fig 6E). It remains to be determined whether they directly bind to the F3 region.

In summary, our study identifies the conserved miRNA miR-34, together with several other miRNAs, as modulators of innate immunity in Drosophila. In particular, we show that miR-34 achieves its immune-modulating function, at least in part, by repressing the expression of two novel target genes (Dlg1 and Eip75B). In addition, our study reveals a mutual repression between miR-34 expression and ecdysone signaling, and identifies miR-34 as a crucial node in the intricate interplay between ecdysone signaling and innate immunity. Lastly, we identify key cis-regulatory genomic elements and trans-acting transcription factors required for optimal ecdysone-mediated repression of miR-34. Taken together, our study enriches the repertoire of immune-modulating miRNAs in animals, and provides new insights into the interplay between steroid hormone signaling and innate immunity.

Materials and Methods

Statistical analyses

All statistical analyses in this manuscript were performed using biological replicates and the sample number (n) is shown for each dataset in the corresponding legend. Most analyses were performed using student t-test, except for survival experiments, which involved the log rank test. Unless noted otherwise, data is shown in this manuscript as mean + standard errors of the mean (SEM), * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
DNA constructs and antibodies

Dlg1 cDNA expression construct: a DNA fragment encoding Flag-Dlg1 was amplified by PCR and cloned into pRmHa-3 using SacI and BamHI restriction sites. Reporter construct carrying the UTR sequences from the dlg1 cDNA: a dlg1 3’ UTR fragment was amplified by PCR and cloned into pRmHa-3-Renilla [84] using BamHI and SalI sites. To generate mutations in the candidate miR-34-binding site, the afore-mentioned reporter construct was subject to site-directed mutagenesis using Phusion Site-Directed Mutagenesis Kit (Thermo Fisher) according to the manufacturer’s instructions. Reporter constructs containing candidate miR-34-binding sites identified in the Eip75B ORF: pairs of oligos containing triple copies of individual sites (wildtype or mutant) were annealed and cloned into pRmHa-3-Renilla using BamHI and SalI sites. To generate various miR-34 enhancer-firefly luciferase plasmid, Selected regions were PCR amplified from genomic DNA, cloned into pCR8-TOPO-GW (Invitrogen), and shuttled to pGL3_GW_luc+ [76] by LR clonase II recombination (Invitrogen). Expression construct for miR-34: a ~500 bp DNA fragment encoding pri-miR-34 was amplified by PCR and cloned into pRmHa-3 using EcoRI and BamHI restriction sites. All constructs were verified by Sanger sequencing. The pMT-Eip75B-PC expression construct was a kind gift from Dr. Edward Dubrovsky [85]. Antibodies employed in this study include Anti-Eip74EF and anti-Eip75B antibodies (gift from Dr. Carl Thummel), anti-Dlg1 and anti-BrC antibodies (Hybridoma Bank).

dsRNA synthesis

Templates for generating dsRNAs were either requested from the DRSC (flyRNAi.org) or amplified by PCR in house. dsRNAs were synthesized using Megascript in vitro transcription kit (Ambion) and purified using RNeasy column (Qiagen).

Drosophila genetics and infection

Fly stocks are maintained on a standard fly food. To generate flies over-expressing select miRNAs, we crossed UAS-miRNA transgenic lines with the da-Gal4; tub-Gal80ts composite line. A control cross was set up between a shRNA transgenic line, which expresses an artificial sh-gfp RNA embedded in the miR-1 cassette. To minimize lethality at early developmental stages due to the requirement for select miRNAs in development, fly crosses were kept at permissive temperature (18˚C) until adult progeny of the appropriate genotype emerge. Subsequently the progeny was shifted to restrictive temperature (29˚C) for 5 days to allow for miRNA transgene expression. For in vivo knockdown experiments, a similar crossing scheme was employed except that the UAS-shRNA lines were used instead of the UAS-miRNA lines. The npr6 allele of BrC (BDSC stock #36562) was employed in experiments in Fig 5. For fly infection experiments, male progeny of the appropriate genotype were either left untreated (as control) or pricked with a sharp needle previously dipped in a concentrated suspension of either the Gram-negative bacteria E. coli or the Gram-positive bacteria M. Luteus, and flies were harvested 6 and 24 hours post-infection, respectively. Levels of mRNAs encoding the antimicrobial peptide genes Dipterocin and Drosomycin, and the control RpL32 mRNA were analyzed by RT-qPCR. Additional infection experiments were carried out by injecting 9.2 nl of bacterial suspension or PBS (control) into flies using Nanoject II (Drummond). For fly survival experiment, a concentrated culture of Ecc15 (OD600 ~5); or a concentrated culture of Enterobacter cloacae (OD600 ~5) were injected. Fly survival was monitored daily. To determine pathogen load, a concentrated culture of Ecc15 (OD600 ~5) or an overnight culture of Enterobacter cloacae (OD600 ~0.5) were injected into flies. Subsequently groups of 4 flies were harvested at various time points post Enterobacter cloacae infection (groups of 1 to 3 flies for Ecc15 infection).
and homogenized in 200 μl of sterile PBS. Diluted fly homogenates were plated onto LB plates containing nalidixic acid (Sigma) (for Enterobacter cloacae) or Ampicillin (for Ecc15), colony forming units (CFU) were recorded after 24 hours and CFU per fly is calculated. In experiments involving flies reared in antibiotics-containing food, flies were reared in standard fly food supplemented with antibiotics (100 μg/mL each of ampicillin, kanamycin and doxycycline).

**Bacterial phagocytosis assay**

The assays were performed as previously described [86]. Briefly, 138 nl of pHrodo Red E. coli BioParticles Conjugate (Life Technologies) suspension in PBS was injected into individual flies of appropriate genotype using Nanoject II. Flies were kept at 25˚C for 2 hours before imaging using an Axioskop Zeiss microscope.

**Cell culture, transfection, and RNAi**

Drosophila S2 cells are maintained in Schneider’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). S2 cells stably expressing miR-34 were generated by transfection with pRmHa-3-miR-34 and the selection marker plasmid pH-S-neo using the calcium phosphate method, followed by selection in medium containing 400 μg/mL G418 (Calbiochem). dsRNA treatment was performed as described previously [87–89]. Briefly, ~2 × 10^6 S2 cells were seeded in 6-well plates for 24 h and then transfected with 3 μg of the appropriate dsRNA using the calcium phosphate protocol. Two days later, the cells were harvested, replated in 6-cm plates for 24 h, and then transfected again with another 9 μg of dsRNA. Three days later, the cells were harvested and used in assays. DNA transfection was performed in the same manner as dsRNA transfections, except that only a single round of transfection was performed.

**Peptidoglycan and ecdysone treatment in S2 cells**

Drosophila S2 cells were treated with 1 μM 20-hydroxyecdysone (Sigma) for 24 hours. Subsequently, cells were treated with 10 μg/mL crude LPS prep (Sigma), which contains peptidoglycan (PGN) as a potent inducer of IMD signaling [6], for another 6 hours. Total RNA was extracted and levels of the antimicrobial peptide genes Diptericin, Cecropin, Attacin A, Defensin, Metchnikowin and Drosomycin, and the control RpL32 mRNA were analyzed by RT-qPCR. For miR-34 overexpression experiments, cells were first transfected with a miR-34 expression construct controlled by the metallothionein promoter. One day post-transfection cells were treated with CuSO₄ (250 μM) for 2 days prior to 20-hydroxyecdysone and PGN treatment.

**Reporter assay**

For luciferase reporter assays, transfections were performed in a 96-well format and the amount of cells and DNA constructs were scaled down accordingly. Briefly, ~5 × 10^5 S2 cells were seeded in 24-well plates the day before transfection. For miR-34 target site reporter assays, 150 ng of the Renilla luciferase reporter gene containing wild type or mutant miR-34-binding site, 25 ng of a firefly luciferase reporter construct [84], and 600 ng of either the miR-34 expression construct or the empty vector were transfected into these cells. Two days later, cells were treated with 250 μM copper sulfate for 24 hours and the reporter activity was measured using the Dual-Glo system (Promega). For data processing, Renilla/firefly ratio was calculated and normalized against the control sample (wildtype reporter in the absence of miR-34).
To identify the regulatory elements required for ecdysone-mediated repression of miR-34, 100 ng of miR-34 enhancer-firefly luciferase plasmid and 100 ng of pol III-Renilla luciferase or 20 ng of actin-Renilla luciferase were transfected into cells. Two days later, cells were treated with 1 μM 20-hydroxyecdysone (Sigma) for 24 hours and luciferase activity was measured. For data processing, first, the firefly/Renilla ratio was calculated; next changes in the F/R ratio in cognate set of samples upon 20-HE treatment \([\text{F/R ratio}_{\text{post 20-HE}} / \text{F/R ratio}_{\text{no 20-HE}}]\) was calculated and normalized to the control sample (transfected with a firefly luciferase reporter gene driven by the regulatory region derived from the traffic jam gene, which is not responsive to ecdysone treatment).

Co-immunoprecipitation

Various combinations of expression constructs for Flag-Dlg1, together with T7-IMD or T7-Kenny were transfected into S2 cells. Two days post-transfection, cells were treated with 250 μM CuSO4 to induce transgene expression and harvested another 24 hrs later. Cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM orthovanadate) supplemented with protease inhibitor cocktail (Roche). Cleared total lysates were immunoprecipitated with anti-Flag antibodies (Sigma). Both input and immunoprecipitated samples were analyzed by SDS-PAGE followed by immunoblotting with antibodies against the Flag (Sigma) or T7 (Novagen) epitopes.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously with minor modifications [90]. Briefly, Cells were fixed by cross-linking with a final concentration of 1.42% formaldehyde for 15min at room temperature and then quenched with 125 mM glycine. After washing with cold PBS twice, the cells were lysed with IP buffer (150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1.0% Triton X-100, 50 mM Tris-HCl pH 7.5). The clarified lysate was subject to sonication. The chromatin was then sheared to fragments of 200–1000 bp and cleared of debris by centrifugation. The chromatin is used for ChIP using anti-BrC antibody (Hybridoma Bank). After washing in IP buffer, the precipitated ChIP DNA was eluted, the cross-links were reversed by incubation at 68˚C for 2h (or overnight). DNA fragments then were purified from the isolated protein-DNA complex and analyzed by qPCR.

RT-qPCR

Total cellular RNA was isolated with TRIzol (Invitrogen) and reverse transcription was carried out using QuantiTect Reverse Transcription kit (Qiagen). Quantitative PCR was performed using the iQ SYBR-green reagents on a CFX96 Real-Time PCR Detection System (Bio-Rad). The efficiency of various primer pairs was determined using serial dilutions of a standard template (RT samples from naïve or 20-HE treated S2 cells). Fold changes in RNA levels were calculated using the ΔΔCt method for primer pairs with efficiencies of 90–111%. Note that fold changes in the dlg1 mRNA were determined by calculating absolute levels of dlg1 and RpL32 using the detected primer efficiency.

Northern blotting

Northern blotting was performed as previously described [91, 92]. In brief, total cellular RNA was isolated with TRIzol (Invitrogen). Samples of 15 μg RNA were separated on 15% denaturing polyacrylamide gels and transferred to Hybond-N+ membranes (Amersham Biosciences) in 1X TBE buffer. Small RNAs were UV crosslinked to the membranes, and the membranes
were prehybridized in hybridization buffer for 2 h. DNA probes complementary to the appropriate strands were 5' radiolabeled and incubated with membranes overnight at 37°C. Membranes were washed twice in 1X SSC with 0.1% SDS at 42°C, and then exposed to Phosphorimager screens for 12–48 h. Membranes were stripped by the addition of boiling 0.1% SDS solution and incubated for 30 min.

**RNA-Seq data analysis**

RNA-Seq datasets containing ~25 million 2 x 90 bp reads were generated from control S2 cells or cells over-expressing miR-34 using an Illumina HiSeq 2000 system. The average insert size for the RNA-seq library was ~200 bp. Pair-end reads were mapped to the *D. melanogaster* genome (UCSC dm3) and to the Flock House virus genome using Bowtie2 (v2.1.0) with TopHat (v2.0.8b) [93]. Differentially expressed transcripts and genes were identified using Cufflinks (v2.1.1) [94].

**Oligonucleotides**

See S3 Table.

**Supporting Information**

**S1 Fig. Depletion of Drosha in vivo impacts innate immunity signaling.** Total RNA was isolated from male flies carrying the ubiquitously expressed da-Gal4 driver and a shRNA construct targeting Drosha (da>Drosha shRNA). Steady-state levels of mRNAs encoding Drosha (A), the AMPs Diptericin and Drosomycin (Drs), as well as levels of the primary bantam miRNA transcript (pri-miR-bantam) (B) were measured by qRT-PCR, and normalized to levels of the Rpl32 mRNA. RNA isolated from da>gfp shRNA males serves as negative control (n≥3; mean + SD).

(TIF)

**S2 Fig. Over-expression of miR-34 dampens the Toll innate immunity signaling pathway.** Flies over-expressing miR-34 were infected by *M. luteus* via septic injury. After one day, RNA was extracted and levels of the Drosomycin (Drs) mRNA was measured by RT-qPCR and normalized to the control RpL32 mRNA (n≥3).

(TIF)

**S3 Fig. Over-expression of miR-34 in S2 cells impact AMP gene expression.** A S2 cell line was established that stably expresses miR-34 under the control of the copper-inducible metallothionein promoter. Cells were treated with ecdysone and a combination of CuSO₄ (to activate the metallothionein promoter) and PGN. Total RNA was isolated and levels of mRNAs encoding various antimicrobial peptides, including Attacin A, Cecropin, Defensin and Metchnikowin were measured by RT-qPCR and normalized to the RpL32 mRNA (mean + SD; n = 3).

(TIF)

**S4 Fig. Over-expression of miR-34 in S2 cells activates pirk expression.** The same set of RNA samples used in S3 Fig was subject to RT-qPCR analysis to examine levels of the *pirk* mRNA (mean + SD; n = 3).

(TIF)

**S5 Fig. miR-34 overexpression or deficiency do not significantly impact bacterial phagocytosis.** *miR-34* overexpression (ox) and knockout (ko) flies were injected with a suspension of pHrodo Red *E. coli* BioParticles Conjugate in PBS. Flies were kept at 25°C for 2 hours before
imaging. Flies expressing sh-gfp (ctr) and ko flies carrying a miR-34 rescue construct (Res), respectively, serve as controls. At least 9 flies per genotype were analyzed and a representative image from each genotype is shown.

S6 Fig. Identification of candidate miR-34 target genes. A Venn diagram shows the number of candidate miR-34 target genes predicted by TargetScan or PicTar, as well as the number of genes that display a decrease in mRNA levels upon miR-34 over-expression in S2 cell. A total of 27 genes were identified that scored positive in all three assays.

S7 Fig. The Eip75B transcript harbors a second miR-34-binding site. (A) Reporter constructs were generated that carry either a wildtype (WT) or mutant (mut) miR-34 binding site derived from the Eip75B ORF. Seed region of miR-34 was highlighted in green. (B) The reporter constructs were transfected into S2 cells together with or without a miR-34 expression construct, and reporter activities were measured (n = 3).

S8 Fig. EcR and BrC are required for optimal ecdysone-mediated induction of let-7 expression. S2 cells transfected with various dsRNAs were left untreated or treated with ecdysone (20-HE) at 1 μM for 48 hrs. Total RNA was isolated and levels of the primary let-7 transcript were measured and normalized to the control RpL32 mRNA (n = 3; mean ± SD).

S9 Fig. Knockdown of Srp, Twi and Ap. S2 cells transfected with various dsRNAs were left untreated or treated with ecdysone (20-HE) at 1 μM for 2 days. Total RNA was isolated and levels of the indicated transcripts were measured and normalized to the control RpL32 mRNA (n = 3).

S10 Fig. Levels of the Dlg1 protein display a decrease in response to IMD signaling in S2 cells. S2 cells were treated with dsRNAs targeting various components of IMD signaling (below). Cells were subsequently treated with ecdysone for 24 hrs, and either remained untreated or treated with PGN for an additional 6 hours. Cells were harvested and levels of the Dlg1 protein were measured by immunoblot (A) and quantified (B; n = 3). The Tubulin protein serves as a loading control.

S11 Fig. Levels of the Dlg1 protein display a decrease in response to IMD signaling in vivo. Flies were infected with E. coli using septic injury. Flies were harvested at different times and levels of the Dlg1 protein (A) and miR-34 (B) in headless fly bodies were measured by immunoblot and Northern blot, respectively. The Tubulin protein and 2S RNA serve as loading controls, respectively. (C) Levels of miR-34 were quantified and normalized against the 2S RNA (n = 3, mean ± SD).

S12 Fig. Levels of the dlg1 mRNA display a decrease upon depletion of IMD signaling components in S2 cells. S2 cells were first treated with dsRNAs targeting various components of IMD signaling (below). Cells were subsequently treated with ecdysone for 24 hrs, and either remained untreated or treated with PGN for an additional 6 hours. Cells were harvested and levels of the dlg1 mRNAs were measured by RT-qPCR and normalized against the RpL32 mRNA. Results from two independent experiments are shown in A and B, respectively.
S13 Fig. Levels of the *dlg1* mRNA display a decrease at early time points of ecdysone treatment in S2 cells. S2 cells were treated with ecdysone and harvested at different times. Levels of the *dlg1* and *Diptericin* mRNAs were measured by RT-qPCR and normalized to the control *RpL32* mRNA (n≥3).

(TIF)

S14 Fig. *Suz12* is another *miR-34* target gene relevant to innate immunity signaling. S2 cells treated with a control dsRNA or dsRNA against *Suz12*. Subsequently, cells were either untreated or treated with PGN. Total RNA was isolated and levels of *Diptericin* were measured and normalized to the *RpL32* mRNA (n = 3).

(TIF)

S1 Table. List of miRNAs analyzed for innate immunity phenotype.

(PDF)

S2 Table. List of 27 candidate *miR-34* target genes identified by TargetScan, PicTar and RNA-sequencing. Cells were transfected with various dsRNAs as indicated. After 3 days, cells were treated with ecdysone (20-HE) at 1 μM for an additional 24 hrs. Cells were harvested, total RNA was extracted and levels of *Diptericin* mRNA was measured by qPCR and normalized to *RpL32*. Cells transfected with a dsRNA against the firefly luciferase serve as a baseline control. Also shown are fold changes in the corresponding mRNA levels upon *miR-34* overexpression.

(PDF)

S3 Table. Oligos employed in this study.

(PDF)

Acknowledgments

We thank Dr. Carl Thummel for antibodies against Eip74EF and Eip75B; Dr. Alexander Stark for control and parental reporter constructs for assaying *miR-34* enhancer activity; Dr. Edward Dubrovsky for *Eip75B* expression construct, Dr. Nancy Bonini for *miR-34KO* and *miR-34* rescue lines, Dr. Neal Silverman for the *Ecc15* and *Enterobacter cloacae* strains, and Dr. Norbert Perrimon for the collection of UAS-miRNA transgenic lines. We thank Dr. Georg Vogler for helping with the imaging analysis on phagocytosis. We also thank Drs. Rolf Bodmer, Karen Ocorr and members of the Bodmer, Rana and Zhou groups for discussions, and the anonymous reviewers for their insightful suggestions.

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