Drosophila Myc restores immune homeostasis of Imd pathway via activating miR-277 to inhibit imd/Tab2

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Abstract

Drosophila Myc (dMyc), as a broad-spectrum transcription factor, can regulate the expression of a large number of genes to control diverse cellular processes, such as cell cycle progression, cell growth, proliferation and apoptosis. However, it remains largely unknown about whether dMyc can be involved in Drosophila innate immune response. Here, we have identified dMyc to be a negative regulator of Drosophila Imd pathway via the loss- and gain-of-function screening. We demonstrate that dMyc inhibits Drosophila Imd immune response via directly activating miR-277 transcription, which further inhibit the expression of imd and Tab2-Ra/b. Importantly, dMyc can improve the survival of flies upon infection, suggesting inhibiting Drosophila Imd pathway by dMyc is vital to restore immune homeostasis that is essential for survival. Taken together, our study not only reports a new dMyc-miR-277-imd/Tab2 axis involved in the negative regulation of Drosophila Imd pathway, and provides a new insight into the complex regulatory mechanism of Drosophila innate immune homeostasis maintenance.

Author summary

Innate immunity is the first line of defense against pathogenic microorganisms. Both overactivation and depression of immune response are detrimental to the organism. It is indispensable to regulate the duration and intensity of immune response. In this work, we find that Drosophila Myc (dMyc) as a transcription factor activates the transcription of miR-277 to negatively regulate the Imd pathway via inhibiting the expression of imd/Tab2 gene in the middle and later stage of Drosophila innate immune. dMyc is required to restore Drosophila immune homeostasis for the survival of flies upon infection. Since dMyc is well conserved in animals, our findings will be important in understanding the complex regulatory mechanisms of innate immune responses in animals.
Introduction

Innate immune system plays critical roles in host defending foreign pathogenic microorganisms [1]. *Drosophila melanogaster* is an important model for studying innate immune response in animals. *Drosophila* involves both cellular and humoral mechanisms to produce diverse antimicrobial peptides (AMPs) to resist the invasion of foreign pathogens via innate immune responses [2, 3]. Transcriptional expressions of *Drosophila* AMPs are primarily controlled by Toll and the immune deficiency (Imd) signaling pathways [4, 5]. *Drosophila* mainly utilizes the Imd signaling pathway to resist Gram-negative bacteria infection [6]. Currently, although the activation mechanisms of innate immune response have been well-established, the study on restoration mechanism of innate immune homeostasis remains a major challenge [7].

The intensity and duration of *Drosophila* immune response can be positively or negatively regulated at multiple layers [8]. For example, STING and sick can activate *Drosophila* Imd innate immune response via upregulating the expression of the NF-κB transcription factor Relish [9, 10]. The E3-ligase inhibitor of apoptosis 2 (Iap2) could activate Dredd expression to positively regulate the Imd immune response [11, 12]. Furthermore, Imd-mediated immune response can be negatively regulated by some immune suppressors, such as WntD, Die, PGRP-LF, pirk, dUSP36, CYLD, Dnr1, DRYBP and Caspar [13–23], which can prevent the excessive activation of *Drosophila* Imd pathway to maintain innate immune homeostasis. In addition, many miRNAs have been reported to participate in fine-tuning *Drosophila* Imd immune response positively or negatively. Studies have shown that *Drosophila* miR-8 and miRNA let-7 could negatively regulate the Imd pathway [24, 25], whereas miR-34 could positively regulate the Imd pathway [8]. Our previous works have also demonstrated that both miR-9a and miR-981 could negatively regulate *Drosophila* Imd-dependent immune response via directly targeting the AMP gene *Diptericin* (*Dpt*) [26]. Although several regulators involved in *Drosophila* innate immune responses have been identified, the further study of the restoration mechanism of *Drosophila* innate immune homeostasis is still needed.

Myc serves as a broad-spectrum transcription factor to control the expression of a large number of genes for diverse cellular processes, including cell cycle progression, cell growth, proliferation and apoptosis [27–31]. Myc family includes three member of c-Myc, N-Myc, and L-Myc in human [27]. It’s well documented that Myc can function as a proto-oncogene in human cancers [32, 33]. *Drosophila* has only one single *Myc* gene, referred to as *dMyc* or diminutive (*dm*), which is homologous to human c-Myc [34]. Studies have revealed that dMyc could involve in ribosome biogenesis [35], protein synthesis [36, 37], cell-autonomous apoptosis [38, 39], and cell competition [40, 41]. Although Myc can regulate the expression of some miRNAs to participate in immune response in human [42–48], it’s largely unknown whether and how dMyc can regulate miRNA expression to control innate immune response in *Drosophila*.

In this work, we firstly report that dMyc can inhibit the immune response of *Drosophila* Imd pathway by genetic screening. Secondly, we confirm that dMyc directly activate the transcription of miR-277 using Chromatin immunoprecipitation (ChIP)-qPCR analysis and promoter reporter activity system. Thirdly, we find that miR-277 can negatively regulate *Drosophila* Imd signaling response via targeting and downregulating the expression of *imd* and *Tab2-Ra/b*, but not *Tab2-Rc*. We further verify that dMyc could negatively regulate *Drosophila* Imd signaling pathway via activating miR-277 transcription to inhibit imd/Tab2 expression in vivo using dMyc and miR-277 SP co-highexpressed flies. Finally, we provide evidences to show that dMyc could restore immune homeostasis of *Drosophila* Imd pathway to promote the survival of flies upon infection.
**Result**

**dMyc is a negative regulator of *Drosophila* Imd pathway**

*Drosophila* defends against Gram-negative bacteria infection through Imd pathway to produce *Diptericin* (*Dpt*) and other anti-microbial peptides such as *Drosocin*, *Attacin* and *Cecropin A1*. In this work, via screening the fly strains from the Bloomington *Drosophila* library, we found *Gal80*ts-UAS-dmyc flies significantly decrease the expressions of *Dpt*, *Drosocin*, *Attacin* and *Cecropin A1* after infection with gram-negative bacteria *Escherichia coli* (*E. coli*), indicating dMyc regulates the Imd pathway (S1 Fig). Therefore, we here chose the *Dpt* as the representative for further exploring the regulatory role of dMyc in immune response of *Drosophila* Imd pathway. Our results demonstrate that the expression level of *Dpt* in the dMyc high-expressed flies with *E. coli* infection is, respectively, significantly lower than wild-type flies at all five time points (3, 6, 12, 24, 48h) (Fig 1A). The expression level of dMyc exists significant differences within different dmyc mutant fly strains (Fig 1B), and the order of the expression level of *Dpt* in dMyc is dMyc high-expressed flies > dMyc and dMyc-RNAi co-highexpressed flies > wild-type flies > dMyc-RNAi high-expressed flies. Remarkably, the expression level of *Dpt* has no significant difference in these above dmyc mutant flies without infection (Fig 1C), but after infected with *E. coli*, the expression level of *Dpt* in dMyc-RNAi high-expressed flies is significantly higher than dMyc high-expressed flies and the control flies, respectively (Fig 1D). Especially, the expression level of *Dpt* in the dMyc and dMyc-RNAi co-highexpressed flies could be nearly restored to the normal expression level of the control flies (Fig 1D). Taken together, our findings strongly suggest that dMyc is a novel important negative regulator of *Drosophila* Imd pathway.

**dMyc regulates the expression of immune related miRNAs**

To investigate whether and how dMyc negatively regulates Imd pathway via regulating miRNA expression, we used in silico analysis to identify potentially immune related dMyc-miRNA-gene axis (Fig 2A). We collected 63 genes involved in *Drosophila* Imd pathway from the literature data, and downloaded the maturation and upstream promoter sequences of *Drosophila* miRNAs from miRBase (http://www.mirbase.org/) and NCBI (https://www.ncbi.nlm.nih.gov/). The relationships between the 63 genes and miRNAs were next predicted by TargetScan and miRanda. Then miRNAs regulated by dMyc were predicted using the PROMO website and TransmiR 2.0 database.

We found 12 candidate dMyc-regulated miRNAs, which have been identified as differentially expressed miRNAs (DEmiRNAs) between flies with *E. coli* infection and the control from our previous small RNA-seq data [26] (Fig 2A, 2B and 2C). We next constructed these 12 DEmiRNAs high-expressed fly strains. We found that the expression level of *Dpt* is significant lower than the control after *E. coli* infection in miR-10, miR-1012, miR-277, miR-2b-2 and miR-996 high-expressed flies, respectively (Figs 2D and S2). We also investigated the expression relevance between dMyc and these 5 miRNAs, finding only miR-277’s expression level in dMyc high-expressed flies is gone up 1.15 times than the control, but not miR-2b-2, miR-1012, miR-10 and miR-996 (Fig 2E). In contrast, the miR-277’s expression level in dMyc knocked down flies is significant lower than the control (Fig 2F). In addition, two ChIP-seq data for dMyc from ENCODE database (https://www.encodeproject.org/) indicated that dMyc could bind to the upstream promoter sequences of *Drosophila* miR-277 gene (S3 Fig). Taken together, our results suggest that dMyc might regulate miR-277 expression to negatively control Imd signaling pathway.
Fig 1. The dMyc functions as a negative regulator of Drosophila Imd pathway. (A) The transcript level of Dpt in dMyc highexpressed flies was measured at 0, 3, 6, 12, 24 and 48 h upon E. coli infection. (B) The expression level of dMyc was examined in control flies (Gal80ts; Tub-Gal4/+), dMyc high-expressed flies (Gal80ts; Tub>UAS-dmyc), dMyc and dMyc-RNAi co-highexpressed flies (Gal80ts; Tub>UAS-dmyc+dmyc-RNAi) and dMyc-RNAi alone high-expressing flies (Gal80ts; Tub>UAS-dmyc-RNAi) before E. coli infection. (C) The expression level of Dpt was detected in control flies (Gal80ts; Tub-Gal4/+) and dMyc high-expressed flies (Gal80ts; Tub>UAS-dmyc), dMyc and dMyc-RNAi co-highexpressed flies (Gal80ts; Tub>UAS-dmyc+dmyc-RNAi) and dMyc-RNAi alone high-expressing flies (Gal80ts; Tub>UAS-dmyc-RNAi) before E. coli infection.
dMyc directly activates the transcription of miR-277

To further study how dMyc regulates miR-277, we performed an analysis for the upstream promoter sequences of miR-277 and found that miR-277 gene contains 2 transcriptional start sites (TSSs) [49] (Fig 3A). The upstream sequences of these two TSSs have the promoter activity, and the promoter activity of TSS1 upstream sequence is stronger than the TSS2 (Fig 3B). Lipopolysaccharide (LPS) stimulation could enhance these two promoter activities to increase miR-277 expression (Fig 3B). Furthermore, dMyc could enhance the luciferase activities of these two promoter regions, and the promoter activity of TSS1 upstream sequence is consistently stronger than that of TSS2 (Fig 3C). ChIP-qPCR assay showed that dMyc is enriched at three candidate regions in the promoter sequences, and the neighbor region of TSS1 (ChIP1) is most enriched (Fig 3D). Taken together, our results suggest that dMyc activates miR-277 transcription via directly binding to its promoter.

miR-277 inhibits the expression of imd and Tab2-Ra/b

To further determine how miR-277 regulates the Imd pathway, first, we examined the expression level of Dpt in miR-277 high-expressed flies compared to the control group flies upon infection. The result showed that the expression level of Dpt in miR-277 high-expressed flies is significantly down-regulated at 3, 6 and 12 h post-infection compared with the control (Fig 4A). Moreover, the miR-277 rescue assay showed that this miR-277 and miR-277 sponge co-highexpressed flies could restore the expression level of miR-277 to the normal level (Fig 4B). Without infection, the expression level of Dpt has no significant difference between miR-277 mutant flies and the control group flies (Fig 4C). Remarkably, the expression level of Dpt could be recovered to the comparable level of the control group in miR-277 and miR-277 sponge co-highexpressed flies after E. coli infection (Fig 4D). Taken together, these results confirm that miR-277 negatively regulates the Imd pathway.

To further explore how miR-277 inhibits the Imd pathway, we predicted the potential target genes of miR-277 using targetScan and miRanda. We found that miR-277 could target to the 3'UTR of imd and Tab2 Ra/b transcripts (Fig 5A and 5B). imd and Tab2 Ra/b are key components of the Imd pathway in Drosophila [6, 50]. As expected, the expression levels of Dpt in both imd-RNAi and Tab2-RNAi mutant flies are significantly lower than the control upon E. coli infection (S4A and S4B Fig). Consistently, compared with the control, the expression levels of both imd and Tab2 are also significantly down-regulated in flies with high-expressed miR-277 at 3 h, 6 h and 12 h post infection, respectively (Fig 5C and 5D). In addition, the expression levels of imd and Tab2 in miR-277 and miR-277 sponge co-highexpressed flies could be restored to the level of the control group (Fig 5E and 5F). Our findings suggest that miR-277 could inhibit the expression of both imd and Tab2 in vivo.

To further evaluate the direct targeting relationship between miR-277 and imd as well as Tab2, we carried out the Luciferase Reporter Assay in Drosophila S2 Cell. The results showed that compared with the pAc empty vector, miR-277 could significantly reduce the activity of the luciferase reporter containing the 3'UTR of imd and Tab2 Ra/b, but not Tab2 Rc (Fig 5G and 5H). Furthermore, we performed the target site mutation in the 3'UTR of imd and Tab2 Ra/b, finding that the reporter activity of imd and Tab2 Ra/b could be nearly restored to the normal level in these cells with co-transfected miR-277 expression vector and 3'UTR mutant.
Fig 2. The screening of dMyc regulating Drosophila immune related miRNAs. (A) The flowchart showed the process of obtaining a batch of potential immune-related dMyc-miRNAs-Genes axes using bioinformatics tools. (B) The Venn diagram exhibited that the intersection of miRNAs that dMyc
reporters of imd and Tab2 Ra/b (Fig 5G and 5H). Taken together, our results suggest that miR-277 directly targets the 3'UTR of imd and Tab2 Ra/b.

dMyc negatively regulates Drosophila Imd pathway via activating miR-277 to inhibit imd/Tab2

To further ascertain whether dMyc can regulate the Drosophila immune response via activating the transcription of miR-277 in vivo, we constructed the dMyc and miR-277 SP co-highexpressed mutation flies. We found that the expression level of miR-277 in the dMyc and miR-277 SP co-highexpressed flies is significantly lower than the dMyc highexpressed flies, and is nearly restored to the control level (S5A and S5B Fig). Without infection, the expression level of Dpt shows no significant difference in aforesaid mutant flies, and the expression level of Dpt in this dMyc and miR-277 SP co-highexpressed flies is significantly higher than the dMyc highexpressed flies upon infection (Fig 6A and 6B). In addition, the expression level of Dpt in the dMyc and miR-277 SP co-highexpressed flies at 6 h post infection could be restored to 55% of the control level, and is nearly 3 times than the dMyc highexpressed flies (Fig 6B). The expression levels of imd and Tab2, respectively, are also restored to 83% and 58% of the control level, and are 2.1 and 1.8 fold than the dMyc highexpressed flies, respectively (Fig 6C and 6D). Taken together, our data suggest that dMyc negatively regulates Drosophila Imd immune response via activating miR-277 transcription to inhibit imd/Tab2 expression.

dMyc controls Drosophila Imd immune homeostasis

dMyc could negatively regulate Drosophila Imd immune response, indicating dMyc could be involved in the maintenance of Imd immune homeostasis. To test this point, we further monitored the dynamic expressions of Dpt, dMyc, miR-277, imd and Tab2 in this wild-type flies at 0, 3, 6, 12, 24 and 48 h after E. coli infection. Our results showed that the expression level of Dpt is increasing before 3 h and reaching its peak expression level at 12 h, then gradually decreased to the basal level post-infection (Fig 7A). In contrast, the expression level of dMyc is decreased before 6 h, implying that the expression of dMyc is inhibited for avoiding the inadequate of immune response in the early stage of E. coli infection (Fig 7B). The expression level of dMyc is markedly up-regulated at 12 h post-infection, and subsequently is restored to the pre-infection level (Fig 7B). Moreover, the expression pattern of miR-277 is very similar with that of dMyc, whereas the expression patterns of both imd and Tab2 are opposites of that of dMyc and miR-277 (Fig 7C and 7D). Taken together, we propose that dMyc could play a key role in restoring Drosophila Imd immune homeostasis post infection.

Immune homeostasis post infection is essential to organisms. We hypothesized that dMyc could protect Drosophila from damage caused by over-activation of immune response. To test this, we further investigated the survival rate of dMyc high-expressed flies and the control (Gal80Ts; Tub-Gal4/+ ) flies without infection and with PBS as well as the Gram-negative bacteria Enterobacter cloacae (E. cloacae) infection, respectively (Fig 8). We found that the survival rate of dMyc high-expressed flies is significantly lower than the control group both in the absence of infection and after infection with PBS. However, the survival rate of dMyc high-expressed flies is significantly higher than the control group after infection with E. cloacae.
Fig 3. dMyc activates the transcription of miR-277. (A) According to the two TSSs of the miR-277 promoter region, the $P_{TSS1}$ (green line) and $P_{TSS2}$ (orange line) region were selected to perform promoter activity analysis, while the ChIP1, ChIP2 and ChIP3 sequences (purple line) were selected to design the primers for ChIP-qPCR assay.
These results seem to support the important role of dMyc in negatively regulating the Imd pathway for immune homeostasis, which is essential for fly survival. Taken all results together, we proposed a molecular mechanism by which dMyc plays an important role in Drosophila Imd immune homeostasis (Fig 9). On the one hand, down-expressed dMyc could down-regulate miR-277 expression to ensure the elevated expression of imd and Tab2 at the early stage of E. coli infection to promote the expression of Dpt against pathogenic bacteria. On the other hand, to prevent the overactivation of Imd immune response, over-expressed AMP Dpt induces dMyc expression to activate the expression of miR-277 for down-regulating the expression of imd and Tab2 to reduce Dpt expression, which restores Imd immune response to homeostasis to protect Drosophila from damage caused by overactivation of immune response, and improve the survival of Drosophila.

Discussion

The Drosophila innate immune system plays critical roles in defending invading pathogens. Depression and overactivation of innate immune responses are both harmful for Drosophila. Thus, the Drosophila innate immune system must gain an unknown mechanism to resist pathogen challenges without overactivation of innate immunity. Although studies have revealed that the Drosophila innate immune response could be controlled by a series of negative or positive regulators at transcriptional and post-transcriptional levels [8, 24–26, 51, 52], the mechanism of maintaining immune homeostasis is largely unknown. In this study, we reveal a new dMyc-miR-277-imd/Tab2 axis to play an important role in negatively regulating Imd pathway, and provide a mechanistic insight into immune homeostasis in Drosophila.

We found that high-expressed dMyc led to decrease of Dpt expression, conversely knock-down dMyc resulted in increase of Dpt expression (Fig 1B), indicating that dMyc act as a negative regulator of Drosophila Imd pathway. Previous studies have revealed that human Mycs play key roles in activating both innate and adaptive immune cells to defense invading pathogens [1, 53, 54]. Functions of Myc are evolutionarily conserved between fruit fly and vertebrate [55]. The fruit flies and vertebrate proteins can substitute for each other to the extent [56]. Thus, our study provides an important insight into illuminate the conservative immune regulation function of Myc between Drosophila and human.

Human Mycs, as important transcriptional factors, not only can control the expressions of a large number of protein-coding genes, but can regulate the expressions of many miRNAs [57, 58]. In this work, we further identified miR-277 as a target gene of dMyc (Fig 3), and found miR-277 could negatively regulate Dpt expression by targeting imd and Tab2 in Drosophila Imd immune responses (Fig 6). Previous studies have indicated that imd and Tab2 are specifically required for the immune activation of Drosophila Imd signaling pathway [6, 59]. Thus, our results suggest that dMyc might negatively regulate Drosophila Imd immune response via activating miR-277 transcription to inhibit imd/Tab2 expression. Previous reports have indicated that miR-277 not only can control branched-chain amino acid catabolism, affect lifespan [60] and wing imaginal discs development of Drosophila [61], but modulate the Neurodegeneration Caused by Fragile X Premutation rCGG Repeats [62]. Whereas, our present results demonstrate that miR-277 is a new negative regulator involved in Drosophila Imd signaling pathway. Especially, the Tab2 is an alternative splicing gene, which contains three
Fig 4. miR-277 negatively regulates Drosophila Imd pathway immune response. (A) The expression level of Dpt was detected in miR-277 highexpressed fly strains (Gal80\(^{ts}\); Tub\( \geq \)miR-277) at 0, 3, 6, 12, 24 and 48 h upon E. coli infection. (B) The expression level of miR-277 was measured in the control flies, this miR-277 highexpressed flies, and this miR-277 and miR-277 sponge co-highexpressed flies before E. coli infection. The transcriptional level of Dpt was determined before (C) and after (D) E. coli infection.

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Fig 5. miR-277 inhibits the expression of \textit{imd} and Tab2-Ra/b \textit{in vivo} and \textit{in vitro}. These potential binding sites of miR-277 in the 3’UTR of \textit{imd} (A) and Tab2-Ra/b (B) were predicted by targetScan and miRanda software. These point mutations at the 3’UTR target sites base pairing to the seed sequence of miR-277.
miR-277 were performed. These expression level of imd (CE) and Tab2 (DF) were respectively tested in miR-277 high-expressing flies and miR-277 and miR-277 sponge co-highexpressed flies. The corresponding luciferase activity of the report plasmids without or with mutation sites was determined in Drosophila S2 cell on a Dual luciferase assay (GH).

transcripts, i.e. Tab2 Ra, Tab2 Rb and Tab2 Rc, of which Tab2 Ra and Tab2 Rb’s 3’UTR is identical. Here, our results showed that miR-277 can target to the 3’UTR of Tab2 Ra and Tab2 Rb transcripts, but not Tab2 Rc (Fig 5). This result suggests that miRNA might play a critical role in selectively regulating the expression of alternative splicing gene in the post-transcriptional level.

Innate immune is a rapid and short immune response process, and inactivation or overactivation of innate immune responses can result in the normal tissue damage [63–66]. We reported the tightly coordinated expression of dmyc, miR-277, imd, Tab2 and Dpt, suggesting that dMyc as a novel negative regulator primarily prevents the over-activation of Drosophila Imd immune response at this middle and later stage of E. coli infection, and helps Drosophila restore to a new immune homeostasis.

Recently, overexpression of dMyc has been reported to be able to significantly diminish Drosophila adult longevity, which might is due to over-expressed dMyc greatly resulting in genome instability [67]. In addition, studies have indicated that down-regulating expression level of c-Myc can significantly increase mice longevity due to heterozygosity [27, 68]. However, in our present study, we found that the overall survival rate of dMyc high-expressed adult male flies is similar with the control group at the early stage (0~5h) after E. cloaca infection (Fig 8). Whereas, the survival rate of dMyc high-expressed adult male flies was significantly higher than the control group after 5 h post infection (Fig 8). Taken together, our findings suggest that dMyc contributes to the survival of flies likely via preventing over-activation of innate immune responses to avoid excessive damage of many tissues.

Conclusions

In this work, we identify dMyc as a novel negative regulator of Drosophila Imd pathway. Mechanically, dMyc positively activate miR-277 transcription, to target the 3’UTR of imd and Tab2-Ra/b to inhibit their expression, leading a new immune homeostasis. Our present results provide a new comprehensive understanding on the complex regulatory mechanism of maintaining innate immune homeostasis in Drosophila.

Materials and methods

Fly stocks

Flies were obtained from the Bloomington Drosophila Stock Center: UAS-dmyc (#7118); UAS-miR-277 (#36559); UAS-miR-277pOng (#61408). These Fly lines carrying UAS-RNA interference (RNAi) constructs were obtained from the Tsinghua Fly Center: dmycRNAi (#47953); imdRNAi (#31706); Tab2RNAi (#24667). As well as previously purchased tubulin-Gal80Ts;TM2/TM6B (#7019) and tubulin-Gal4/TM3, Sb1, Ser1 (#5138), all flies were raised on maize malt molasses food in a light-dark (12-hr cycle) incubator at 25˚C and 60% humidity. Flies were shifted to 30˚C 24 h prior to and then during infection for UAS-protein/UAS-miR-277pOng or UAS-proteinRNAi overexpression experiments.

Bioinformatic analysis

These mature sequences of Drosophila miRNAs were downloaded from miRBase (http://www.mirbase.org/). 3’UTRs of 63 Imd pathway-related genes and promoter sequences of Drosophila
dMyc negatively regulates *Drosophila* Imd pathway via activating miR-277 to inhibit imd/Tab2 expression.

(A) The expression level of Dpt was examined in control flies, dMyc high-expressed flies, dMyc and miR-277 sponge co-highe xpressed flies (Gal80°; Tub>UAS-dmyc+miR-277 SP) before *E. coli* infection, respectively. The expression levels of Dpt (B), imd (C) and Tab2 (D) were detected and compared in dMyc highexpressed flies and dMyc and miR-277 co-highexpressed flies at 6 h following *E. coli* infection, respectively.

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**Fig 6.** dMyc negatively regulates *Drosophila* Imd pathway via activating miR-277 to inhibit imd/Tab2 expression. (A) The expression level of Dpt was examined in control flies, dMyc high-expressed flies, dMyc and miR-277 sponge co-highexpressed flies (Gal80°; Tub>UAS-dmyc+miR-277 SP) before *E. coli* infection, respectively. The expression levels of Dpt (B), imd (C) and Tab2 (D) were detected and compared in dMyc highexpressed flies and dMyc and miR-277 co-highexpressed flies at 6 h following *E. coli* infection, respectively.

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miRNAs were extracted from the fruit fly genome in FlyBase (http://flybase.org/) and NCBI (https://www.ncbi.nlm.nih.gov/). These relationships between mature miRNAs and 3'UTR of genes were predicted using two miRNA target prediction programs with default parameters, i.e. TargetScan (www.targetscan.org/fly_12/) [69] and miRanda v3.3a tool downloaded from microRNA.org-Targets and Expression [70, 71]. Whilst these sites of transcription factor (dMyc) binding at these promoter sequences of miRNAs were predicted through PROMO website [72, 73] and TransmiR 2.0 database (http://www.cuilab.cn/transmir).

Infection and survival experiments of adult flies

Three to four-day-old adult male flies were used for septic injury experiments. Control and high-expressed or knockdown gene/miRNA flies were infected by E. coli, which is a widely
used bacterial strain that can activate the Imd-mediated immune response to induce the expression of Diptericin. Infection experiments were performed by pricking the thorax of the flies with a pulled glass capillary carrying *E. coli* inoculant using a Nanoject apparatus (Nanoliter 2010, WPI). Next, flies were collected at specified time-points for subsequent experiments. Survival to infection is the most holistic approach to assess these defects in immune response [74]. For the survival experiment, flies were infected with a concentrated culture of *E. cloacaee* by pricking as above, and then the survival situation of flies was detailedly recorded for 5 days.

**RNA extraction and RT-qPCR**

Total RNAs were isolated from these treated adult flies using TRizol Reagent (Invitrogen) following the instructions. For RT-PCR, a first-strand cDNA synthesis kit (Vazyme, China) was used to prepare the cDNA. These stem-loop primers were synthesized for reverse transcription to generate the specific stem-loop cDNA of miRNA. Quantitative PCR reactions were performed using AceQ SYBR Green Master Mix (Vazyme, China) on the ABI StepOne Plus Real-
Time PCR System (Applied Biosystems, USA). The expression levels of mRNA and miRNA were normalized to the control rp49 and U6 snRNA, respectively. All experiments were in triplicate. The relative $2^{ΔΔCT}$ method was used for data analysis [75]. All primers used in this analysis were listed in S1 Table.

**Cell culture and immune stimulations**

*Drosophila* S2 cells were maintained at 28˚C in Schneider’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). For immune stimulation, cells were incubated with 10μg/ml commercial LPS from *E. coli* 055:B5 (Sigma, St. Louis, MO), which is characteristic components of the cell wall of Gram-negative bacteria, for 6 h [76, 77].

**Chromatin immunoprecipitation (ChIP)**

For ChIP experiment, Cells were fixed by cross-linking with a final concentration of 1% formaldehyde solution for 10 min at room temperature and then quenched with 125 mM glycine for 5 min. After washing with cold PBS containing a protease inhibitor cocktail and PMSF twice, these cells were lysed with cell lysis buffer and nuclear lysis buffer. The clarified lysate was subject to sonication. The chromatin was then sheared to fragments of 200–500 bp. The chromatin was used for ChIP incubating with Dynabeads protein G (Thermo Fisher Scientific) coated with either an anti-dMyc antibody (P4C4-B10; DSHB) or mouse IgG control antibody.

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**Fig 9. A potential molecular mechanism of dMyc restoring immune homeostasis of *Drosophila* Imd pathway.** Our results suggested a model in which dMyc could restore *Drosophila* Imd immune homeostasis at the middle and late stage of *E. coli* infection. Left diagram: *Drosophila* Imd pathway is activated at the early stage of infection. Right diagram: Most of the bacteria have been eliminated at the middle stage of infection, next highly expressed antimicrobial peptides induce the up-regulated expression of dMyc. Then dMyc transfers into the nuclear to bind the upstream region of *miR-277* to activate *miR-277* transcription. Mature *miR-277* further suppresses *imd* and *Tab2* expression to down-regulate the Imd pathway immune response, and then assists cells restore immune homeostasis at the late stage of infection. Red word: up-regulation; Green word: down-regulation.

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overnight at 4°C on a rotating platform. After repeated washes using a magnetic rack (Thermo Fisher Scientific), dMyc-bound genomic DNA was eluted from Dynabeads, and then the cross-links were reversed at 65°C for 4h (or overnight). DNA fragments then were purified with AxyPrep PCR Cleanup Kit (Axygen). qRT-PCR analysis was performed using the DNA from the Input and ChIP experiments with primers listed in S2 Table. At least three independent experiments were carried out for the miR-277 promoters, as well as for Fibrillarin gene served as a positive control [78].

**Luciferase reporter construction and luciferase assay**

This pri-mir-277 has two transcription initiation sites (TSSs) as reported [49], so we further divided the promoter region of pri-mir-277 into two parts for luciferase promoter analysis. Promoter sequences of miR-277 and CDS of dMyc were amplified by PCR from Drosophila genomic DNA. The DNA fragments were then isolated and inserted respectively into the restriction enzyme digested the promoterless pGL3 Basic and pAc5.1 Vector using T4 DNA ligase. pAc5.1 luciferase reporter constructs carrying the 3’-UTR of either imd or Tab2 with wild-type or mutated sequences of their respective miR-277 target sites were utilized to analyze the effects of the miR-277. All constructs were confirmed by sequencing. All PCR primers for the reporter constructs were listed in S3 Table. Drosophila S2 cells were transfected with each reporter construct for 48 h followed by assessment of luciferase activity. Luciferase activity was then measured with Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions and normalized to the Renilla luciferase activity for each transfected well. Each assay was performed in triplicate.

**Statistical analysis**

All experimental data in this work were collected from three independent biological replicates. All statistical analyses were presented as means ± SEM. Significant differences between the values under different experimental conditions were subjected to two-tailed Student’s t-test. Statistical analysis of fly survival experiments was performed using the log-rank (Mantel-Cox) test. For all tests, $P$ value < 0.05 was considered as statistically significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and ns, no significance vs. the control groups.

**Supporting information**

S1 Fig. The expression level of multiple AMPs in the dMyc high-expressing flies and the control flies. The expression level of Diptericin (A), Attacin (B), Cecropin A1 (C), and Drosocin (D) were measured in the dMyc high-expressing flies and the control flies upon E. coli infection.

(TIF)

S2 Fig. The expression level of Dpt in 7 miRNA highexpressed fly strains. After 7 miRNAs were high-expressed respectively, the Dpt level was determined at 6 h upon E. coli infection. OE: overexpression.

(TIF)

S3 Fig. The bind sites of dMyc on the upstream of miR-277 gene. Two ChIP-seq data for dMyc from ENCODE database were visualized to show the bind sites of dMyc on the upstream of miR-277 gene.

(TIF)
S4 Fig. The expression level of Dpt in this imd-RNAi and Tab2-RNAi highexpressed fly strains. The expression level of Dpt was determined respectively by RT-q PCR in this imd-RNAi (A) and the Tab2-RNAi (B) highexpressed flies upon E. coli infection. (TIF)

S5 Fig. The expression level of dMyc and miR-277 in dMyc and miR-277 sponge co-highexpressed fly strains. The expression levels of dMyc (A) and miR-277 (B) were examined in the control flies, the dMyc highexpressed flies, the dMyc and miR-277 sponge co-highexpressed flies before E. coli infection. (TIF)

S1 Table. Primers used for quantitative RT-PCR (DOCX)

S2 Table. Primers used for ChIP-qPCR (DOCX)

S3 Table. Primers used for transgene vector construction (DOCX)

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References
1. Riera Romo M, Perez-Martinez D, Castillo Ferrer C. Innate immunity in vertebrates: an overview. Immunology. 2016; 148(2):125–39. https://doi.org/10.1111/imm.12597 PMID: 26878338; PubMed Central PMCID: PMC4863567.
2. Hoffmann JA. Innate immunity of insects. Curr Opin Immunol. 1995; 7(1):4–10. https://doi.org/10.1016/0952-7915(95)80022-0 PMID: 7772280.
3. Hultmark D. Immune reactions in Drosophila and other insects: a model for innate immunity. Trends in genetics: TiG. 1993; 9(5):178–83. https://doi.org/10.1016/0168-9525(93)90165-e PMID: 8337755.
4. Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspectives in innate immunity. Science. 1999; 284(5418):1313–8. https://doi.org/10.1126/science.284.5418.1313 PMID: 10334979.

5. Khush RS, Leulier F, Lemaitre B. Drosophila immunity: two paths to NF-kappaB. Trends Immunol. 2001; 22(5):260–4. https://doi.org/10.1016/s1471-4906(01)01887-7 PMID: 11323284.

6. Myllymaki H, Valanne S, Ramet M. The Drosophila imd signaling pathway. J Immunol. 2014; 192(8):3455–62. https://doi.org/10.4049/jimmunol.1303309 PMID: 24706930.

7. Chen CZ, Schaffert S, Fragoso R, Loh C. Regulation of immune responses and tolerance: the microRNA perspective. Immunol Rev. 2013; 253(1):112–28. https://doi.org/10.1111/imr.12060 PMID: 23550642; PubMed Central PMCID: PMC3684622.

8. Xiong XP, Kurfthokt K, Chang KY, Li JL, Ren X, Ni QJ, et al. miR-34 Modulates Innate Immunity and Ecdysone Signaling in Drosophila. PLoS Pathog. 2016; 12(11):e1006034. https://doi.org/10.1371/journal.ppat.1006034 PMID: 27893816; PubMed Central PMCID: PMC5125713.

9. Martin M, Hiroyasu A, Guzman RM, Roberts SA, Goodman AG. Analysis of Drosophila STING Reveals an Evolutionarily Conserved Antimicrobial Function. Cell reports. 2018; 23(12):3537–50 e6. https://doi.org/10.1016/j.celrep.2018.05.029 PMID: 29924997; PubMed Central PMCID: PMC6114933.

10. Foley E O’Farrell PH. Functional dissection of an innate immune response by a genome-wide RNAi screen. PLoS Biol. 2004; 2(8):E203. https://doi.org/10.1371/journal.pbio.0020203 PMID: 15221030; PubMed Central PMCID: PMC434151.

11. Valanne S, Kleino A, Myllymaki H, Vuoristo J, Ramet M. Iap2 is required for a sustained response in the Drosophila imd signaling pathway. J Immunol. 2014; 192(8):3455–62. https://doi.org/10.4049/jimmunol.1303309 PMID: 24706930.

12. Basbous N, Coste F, Leone P, Vincentelli R, Royet J, Kellenberger C, et al. The Drosophila peptidoglycan-recognition protein LF blocks PGRP-LC and IMD/JNK pathway activation. Cell host & microbe. 2008; 3(5):293–303. https://doi.org/10.1016/j.chom.2008.04.002 PMID: 18474356.

13. Labib S, Meignin C, Imler JL. WntD and Diedel: Two immunomodulatory cytokines in Drosophila. Fly (Austin). 2016; 10(4):187–94. https://doi.org/10.1080/19336934.2016.1202387 PMID: 27314646; PubMed Central PMCID: PMC5036923.

14. Persson C, Oldervi S, Steiner H. Peptidoglycan recognition protein LF: a negative regulator of Drosophila immunity. Insect Biochem Mol Biol. 2007; 37(12):1309–16. https://doi.org/10.1016/j.ibmb.2007.08.003 PMID: 17967349.

15. Maillet F, Bischoff V, Vignal C, Hoffmann J, Royet J. The Drosophila peptidoglycan recognition protein PGRP-LF blocks PGRP-LC and IMD/JNK pathway activation. Cell host & microbe. 2008; 3(5):293–303. https://doi.org/10.1016/j.chom.2008.04.002 PMID: 18474356.

16. Basbous N, Coste F, Leone P, Vincentelli R, Royet J, Kellenberger C, et al. The Drosophila peptidoglycan-recognition protein LF interacts with peptidoglycan-recognition protein LC to downregulate the Imd pathway. EMBO Rep. 2011; 12(4):327–33. https://doi.org/10.1038/embor.2011.19 PMID: 21372849; PubMed Central PMCID: PMC3077246.

17. Klein A, Myllymaki H, Kallio J, Vanha-aho LM, Oksanen K, Ulvila J, et al. Pirk is a negative regulator of the Drosophila Imd pathway. J Immunol. 2008; 180(8):5413–22. https://doi.org/10.4049/jimmunol.180.8.5413 PMID: 18390723.

18. Thevenon D, Engel E, Avet-Rochex A, Gotta M, Bergeret E, Tricoire H, et al. The Drosophila ubiquitin-specific protease dusP36/scn targets IMD to prevent constitutive immune signaling. Cell host & microbe. 2009; 6(4):309–20. https://doi.org/10.1016/j.chom.2009.09.007 PMID: 19837371.

19. Tsichritzis T, Gaentzsch PC, Kosmidis S, Brown AE, Skoulakis EM, Ligoxygakis P, et al. A Drosophila ortholog of the human cylindromatosis tumor suppressor gene regulates triglyceride content and antibacterial defense. Development. 2007; 134(14):2605–14. https://doi.org/10.1242/dev.02859 PMID: 17553907.

20. Guntermann S, Primrose DA, Foley E. Dnr1-dependent regulation of the Drosophila immune deficiency signaling pathway. Developmental and comparative immunology. 2009; 33(1):127–34. https://doi.org/10.1016/j.dci.2008.07.021 PMID: 18775745.

21. Aparicio R, Neyen C, Lemaitre B, Busturia A. dRYBP contributes to the negative regulation of the Drosophila Imd pathway. PLoS One. 2013; 8(4):e62052. https://doi.org/10.1371/journal.pone.0062052 PMID: 23596533; PubMed Central PMCID: PMC3626645.

22. Kim M, Lee JH, Lee SY, Kim E, Chung J. Caspar, a suppressor of antibacterial immunity in Drosophila. Proc Natl Acad Sci U S A. 2006; 103(44):16355–63. https://doi.org/10.1073/pnas.0603238103 PMID: 17050695; PubMed Central PMCID: PMC1637587.

23. Kallio J, Leinonen A, Ulvila J, Valanne S, Ezekowitz RA, Ramet M. Functional analysis of immune response genes in Drosophila identifies JNK pathway as a regulator of antimicrobial peptide gene...
expression in S2 cells. Microbes and infection. 2005; 7(5–6):811–9. https://doi.org/10.1016/j.micinf.2005.03.014 PMID: 15890554.

24. Choi IK, Hyun S. Conserved microRNA miR-8 in fat body regulates innate immune homeostasis in Drosophila. Developmental and comparative immunology. 2012; 37(1):50–4. https://doi.org/10.1016/j.dci.2011.12.008 PMID: 22210547.

25. Garbuzov A, Tatar M. Hormonal regulation of Drosophila microRNA let-7 and miR-125 that target innate immunity. Fly (Austin). 2010; 4(4):306–11. https://doi.org/10.4161/fly.4.4.13008 PMID: 20798594; PubMed Central PMCID: PMC3174482.

26. Li S, Shen L, Sun L, Xu J, Jin P, Chen L, et al. Small RNA-Seq analysis reveals microRNA-regulation of the Imd pathway during Escherichia coli infection in Drosophila. Developmental and comparative immunology. 2017; 70:80–7. https://doi.org/10.1016/j.dci.2017.01.008 PMID: 28069431.

27. Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nature reviews Cancer. 2008; 8(12):976–90. https://doi.org/10.1038/nrc2231 PMID: 19029958.

28. Bretones G, Delgado MD, Leon J. Myc and cell cycle control. Biochim Biophys Acta. 2015; 1849(5):506–16. https://doi.org/10.1016/j.bbagrm.2014.03.013 PMID: 24704206.

29. Garcia-Gutierrez L, Delgado MD, Leon J. MYC Oncogene Contributions to Release of Cell Cycle Brakes. Genes (Basel). 2019; 10(3). https://doi.org/10.3390/genes10030244 PMID: 30909496; PubMed Central PMCID: PMC6470592.

30. Cavalheiro GR, Matos-Rodrigues GE, Gomes AL, Rodrigues PM, Martins RA. c-Myc regulates cell proliferation during lens development. PLoS One. 2014; 9(2):e87182. https://doi.org/10.1371/journal.pone.0087182 PMID: 24503550; PubMed Central PMCID: PMC3913586.

31. McMahon SB. MYC and the control of apoptosis. Cold Spring Harb Perspect Med. 2014; 4(7):a014407. https://doi.org/10.1101/cshperspect.a014407 PMID: 24985130; PubMed Central PMCID: PMC4066641.

32. Lutz W, Leon J, Eilers M. Contributions of Myc to tumorigenesis. Biochim Biophys Acta. 2002; 1602(1):61–71. https://doi.org/10.1016/s0304-419x(02)00036-7 PMID: 11960695.

33. Gordan JD, Thompson CB, Simon MC. HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. Cancer cell. 2007; 12(2):108–13. https://doi.org/10.1016/j.ccr.2007.07.006 PMID: 17692803; PubMed Central PMCID: PMC3215289.

34. Prober DA, Edgar BA. Ras1 promotes cellular growth in the Drosophila wing. Cell. 2000; 100(4):435–46. https://doi.org/10.1016/s0092-8674(00)80679-0 PMID: 10693760.

35. Secombe J, Li L, Carlos L, Eisenman RN. The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth. Genes Dev. 2007; 21(6):537–51. https://doi.org/10.1101/gad.1523007 PMID: 17692803; PubMed Central PMCID: PMC3215289.

36. Benassayag C, Montero L, Muller N, Gallant P. Induction of apoptosis by Drosophila Myc. Genesis. 2008; 46(2):104–11. https://doi.org/10.1002/dvg.20373 PMID: 18257071.

37. Moreno E, Basler K. dMyc transforms cells into super-competitors. Cell. 2004; 117(1):117–29. https://doi.org/10.1016/s0092-8674(04)00262-4 PMID: 15066287.

38. de la Cova C, Abril M, Bellosta P, Gallant P, Johnston LA. Drosophila myc regulates organ size by inducing cell competition. Cell. 2004; 117(1):107–16. https://doi.org/10.1016/s0092-8674(04)00214-4 PMID: 15066286.

39. Purvis IJ, Avilala J, Guda MR, Venkataraman S, Vibhakar R, Tsung AJ, et al. Role of MYC-miR-29-B7-H3 in Medulloblastoma Growth and Angiogenesis. Journal of clinical medicine. 2019; 8(8). https://doi.org/10.3390/jcm8081158 PMID: 31382461; PubMed Central PMCID: PMC6723910.

40. Li H, Liu J, Cao W, Xiao X, Liang L, Liu-Smith F, et al. C-myc/miR-150/EPGS axis mediated dysfunction of autophagy promotes development of non-small cell lung cancer. Theranostics. 2019; 9(18):5134–48. https://doi.org/10.7150/thno.34887 PMID: 31410206; PubMed Central PMCID: PMC6691579.

41. Su R, Gong JN, Chen MT, Song L, Shen C, Zhang XH, et al. c-Myc suppresses miR-451 dash, vertica-IYWTAZ/AKT axis via recruiting HDAC3 in acute myeloid leukemia. Oncotarget. 2016; 7(47):77430–43. https://doi.org/10.18632/oncotarget.12679 PMID: 27764807; PubMed Central PMCID: PMC5363596.
Tan H, Poidevin M, Li H, Chen D, Jin P. MicroRNA-277 modulates the neurodegeneration caused by...

Jones CI, Grima DP, Waldron JA, Jones S, Parker HN, Newbury SF. The 5'-3' exoribonuclease Pac...

Winer A, Bodor JN, Borghaei H. Identifying and managing the adverse effects of immune checkpoint...

PMID: 21333734.

Gallant P. Myc function in Drosophila. Cold Spring Harb Perspect Med. 2013; 3(10):a014324. https://doi.org/10.1101/cshperspect.a014324 PMID: 24084813.

Bellosta P, Gallant P. Myc Function in Drosophila. Genes & cancer. 2010; 1(6):542–6. https://doi.org/10.1177/1947601910377490 PMID: 21073235; PubMed Central PMCID: PMC2976539.

Nie Z, Hu G, Wei G, Cui K, Yamane A, Resch W, et al. c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell. 2012; 151(1):68–79. https://doi.org/10.1016/j.cell.2012.08.033 PMID: 23021216; PubMed Central PMCID: PMC3471363.

Lin CY, Loven J, Rahi PB, Paranal RM, Burge CB, Bradner JE, et al. Transcriptional amplification in tumor cells with elevated c-Myc. Cell. 2012; 151(1):56–67. https://doi.org/10.1016/j.cell.2012.08.026 PMID: 23021215; PubMed Central PMCID: PMC3462372.

Zhuang ZH, Sun L, Kong L, Hu JH, Yu MC, Reinaich P, et al. Drosophila TAB2 is required for the immune activation of JNK and NF-kappaB. Cellular signalling. 2006; 18(7):964–70. https://doi.org/10.1016/j.cellsig.2005.08.020 PMID: 16311020.

Esslinger SM, Schwabl B, Helfer S, Michalik KM, Witte H, Maier KC, et al. Drosophila miR-277 controls branched-chain amino acid catabolism and affects lifespan. RNA biology. 2013; 10(6):1042–56. https://doi.org/10.4161/ma.24810 PMID: 23690733; PubMed Central PMCID: PMC3904584.

Rino RM, Grima DP, Waldron JA, Jones S, Parker HN, Newbury SF. The 5'-3' exoribonuclease Pacman (Xrn1) regulates expression of the heat shock protein Hsp70Bc and the microRNA miR-277-3p in Drosophila wing imaginal discs. RNA biology. 2013; 10(8):1345–55. https://doi.org/10.4161/ma.28354 PMID: 23792537; PubMed Central PMCID: PMC3817156.

Tavtigian SV, Ghalamzi H, Nilsson L, et al. Identiﬁcation of 277-regulated genes in melanoma cells. Genes (Basel). 2012; 3(3):188–97. https://doi.org/10.3390/genes3030088 PMID: 23178405.

PMID: 23021215; PubMed Central PMCID: PMC2680477.

Goto A, Matsushita K, Gesellchen V, El Chamy L, Kuttenkeuler D, Takeuchi O, et al. Akirins are highly conserved nuclear proteins required for NF-kappaB-dependent gene expression in drosophila and mice. Nat Immunol. 2008; 9(1):97–104. https://doi.org/10.1038/nii1543 PMID: 18066067; PubMed Central PMCID: PMC1276168.

Psathas JN, Doonan PJ, Raman P, Freedman BD, Minn AJ, Thomas-Tikhonenko A. The Myc-miR-17-92 cluster drives the acquisition of temozolomide resistance in glioblastoma. Brain: a journal of neurology. 2015; 138(Pt 12):3654–72. https://doi.org/10.1093/brain/awv287 PMID: 26450587.

Kleino A, Valanne S, Ulvila J, Kuttenkeuler D, Takeuchi O, et al. Akirins are highly conserved nuclear proteins required for NF-kappaB-dependent gene expression in drosophila and mice. Nat Immunol. 2008; 9(1):97–104. https://doi.org/10.1038/nii1543 PMID: 18066067; PubMed Central PMCID: PMC1276168.

Gallant P. Myc function in Drosophila. Cold Spring Harb Perspect Med. 2013; 3(10):a014324. https://doi.org/10.1101/cshperspect.a014324 PMID: 24086064; PubMed Central PMCID: PMC3784813.

Nie Z, Hu G, Wei G, Cui K, Yamane A, Resch W, et al. c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell. 2012; 151(1):68–79. https://doi.org/10.1016/j.cell.2012.08.033 PMID: 23021216; PubMed Central PMCID: PMC3471363.

Lin CY, Loven J, Rahi PB, Paranal RM, Burge CB, Bradner JE, et al. Transcriptional amplification in tumor cells with elevated c-Myc. Cell. 2012; 151(1):56–67. https://doi.org/10.1016/j.cell.2012.08.026 PMID: 23021215; PubMed Central PMCID: PMC3462372.

Zhuang ZH, Sun L, Kong L, Hu JH, Yu MC, Reinaich P, et al. Drosophila TAB2 is required for the immune activation of JNK and NF-kappaB. Cellular signalling. 2006; 18(7):964–70. https://doi.org/10.1016/j.cellsig.2005.08.020 PMID: 16311020.

Esslinger SM, Schwabl B, Helfer S, Michalik KM, Witte H, Maier KC, et al. Drosophila miR-277 controls branched-chain amino acid catabolism and affects lifespan. RNA biology. 2013; 10(6):1042–56. https://doi.org/10.4161/ma.24810 PMID: 23690733; PubMed Central PMCID: PMC3904584.

Jones CI, Grima DP, Waldron JA, Jones S, Parker HN, Newbury SF. The 5'-3' exoribonuclease Pacman (Xrn1) regulates expression of the heat shock protein Hsp70Bc and the microRNA miR-277-3p in Drosophila wing imaginal discs. RNA biology. 2013; 10(8):1345–55. https://doi.org/10.4161/ma.28354 PMID: 23792537; PubMed Central PMCID: PMC3817156.

Tan H, Poidevin M, Li H, Chen D, Jin P. MicroRNA-277 modulates the neurodegeneration caused by Fragile X premutation rCGG repeats. PLoS Genet. 2012; 8(5):e1002681. https://doi.org/10.1371/journal.pgen.1002681 PMID: 22570635; PubMed Central PMCID: PMC3433002.

Winer A, Bodor JN, Borghaei H. Identifying and managing the adverse effects of immune checkpoint blockade. Journal of thoracic disease. 2018; 10(Suppl 3):S480–S9. https://doi.org/10.21037/jtd.2018.01.111 PMID: 29593893; PubMed Central PMCID: PMC5861268.
64. Arefin B, Kunc M, Krautz R, Theopold U. The Immune Phenoype of Three Drosophila Leukemia Models. G3. 2017; 7(7):2139–49. https://doi.org/10.1534/g3.117.039487 PMID: 28476910; PubMed Central PMCID: PMC5499123.

65. He X, Yu J, Wang M, Cheng Y, Han Y, Yang S, et al. Bap180/Baf180 is required to maintain homeostasis of intestinal innate immune response in Drosophila and mice. Nature microbiology. 2017; 2:17056. https://doi.org/10.1038/nmicrobiol.2017.56 PMID: 28418397.

66. Ragab A, Buechling T, Gesellchen V, Spirohn K, Boutros M. Drosophila Ras/MAPK signalling regulates innate immune responses in immune and intestinal stem cells. The EMBO journal. 2011; 30(6):1123–36. https://doi.org/10.1038/emboj.2011.4 PMID: 21297578; PubMed Central PMCID: PMC3061042.

67. Greer C, Lee M, Westerhof M, Spokony R, Vijg J, et al. Myc-dependent genome instability and lifespan in Drosophila. PLoS One. 2013; 8(9):e74641. https://doi.org/10.1371/journal.pone.0074641 PMID: 24043032; PubMed Central PMCID: PMC3765364.

68. Hofmann JW, Zhao X, De Cecco M, Peterson AL, Pagliaroli L, Manivannan J, et al. Reduced expression of MYC increases longevity and enhances healthspan. Cell. 2015; 160(3):47–88. https://doi.org/10.1016/j.cell.2014.12.016 PMID: 25619689; PubMed Central PMCID: PMC4624921.

69. Ruby JG, Stark A, Johnston WK, Kellis M, Bartel DP, Lai EC. Evolution, biogenesis, expression, and target predictions of a substantially expanded set of Drosophila microRNAs. Genome research. 2007; 17(12):1850–64. https://doi.org/10.1101/gr.6597907 PMID: 17989254; PubMed Central PMCID: PMC2099583.

70. Enright AJ, John B, Gaul U, Tuschi T, Sander C, Marks DS. MicroRNA targets in Drosophila. Genome Biol. 2003; 5(1):R1. https://doi.org/10.1186/gb-2003-5-1-r1 PMID: 14709173; PubMed Central PMCID: PMC395733.

71. John B, Enright AJ, Aravin A, Tuschi T, Sander C, Marks DS. Human MicroRNA targets. PLoS Biol. 2004; 2(11):e363. https://doi.org/10.1371/journal.pbio.0020363 PMID: 15502875; PubMed Central PMCID: PMC521178.

72. Messeguer X, Escudero R, Farre D, Nunez O, Martinez J, Alba MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics. 2002; 18(2):333–4. https://doi.org/10.1093/bioinformatics/18.2.333 PMID: 11847087.

73. Farre D, Roset R, Huerta M, Adsuara JE, Rosello L, Alba MM, et al. Identification of patterns in biological sequences at the ALGEN server: PROMO and MALGEN. Nucleic acids research. 2003; 31(13):3651–3. https://doi.org/10.1093/nar/gkg605 PMID: 12824368; PubMed Central PMCID: PMC168011.

74. Neyen C, Bretscher AJ, Binggeli O, Lemaître B. Methods to study Drosophila immunity. Methods. 2014; 68(1):116–28. https://doi.org/10.1016/j.ymeth.2014.02.023 PMID: 24631888.

75. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4):402–8. https://doi.org/10.1067/meth.2001.1262 PMID: 11846609.

76. Leulier F, Parquet C, Pili-Floury S, Ryu JH, Caroff M, Lee WJ, et al. The Drosophila immune system detects bacteria through specific peptidoglycan recognition. Nat Immunol. 2003; 4(5):478–84. https://doi.org/10.1038/nimm922 PMID: 12692550.

77. Kim YS, Han SJ, Ryu JH, Choi KH, Hong YS, Chung YH, et al. Lipopolysaccharide-activated kinase, an essential component for the induction of the antimicrobial peptide genes in Drosophila melanogaster cells. The Journal of biological chemistry. 2000; 275(3):2071–9. https://doi.org/10.1074/jbc.275.3.2071 PMID: 10636911.

78. Orian A, van Steensel B, Deirouj J, Bussemaker HJ, Li L, Sawado T, et al. Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. Genes Dev. 2003; 17(9):1101–14. https://doi.org/10.1101/gad.1006903 PMID: 12695332; PubMed Central PMCID: PMC1960553.