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Identification of putative miRNA involved in *Drosophila melanogaster* immune response

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ABSTRACT

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that post-transcriptionally regulate gene expression in eukaryotes. They are known to play diverse roles in physiological processes such as homeostasis, development, cancer and immune response. In *Drosophila melanogaster* up to 176 miRNAs have been identified; yet, their biological functions remain unknown. Here, we describe an *in silico* screening strategy to identify miRNAs involved in a specific immune signaling pathway that is based on: (i) the potential capability of miRNAs to target mRNAs of a given pathway; (ii) the sequence conservation of miRNAs across species and (iii) the expression profile of miRNAs. Using this strategy, we have defined a subset of seven *Drosophila* miRNAs that are likely to participate in the immune response. Interestingly, some of these miRNAs target peptidoglycan receptor proteins (PGRPs) for which no regulators are known yet. miRNA-mediated regulation may explain how PGRPs are controlled in the immune signaling pathway.

1. Introduction

MicroRNAs (miRNAs) are small (18–24 nucleotides) non-coding RNA molecules that regulate gene expression at post-transcriptional level by repressing messenger RNAs (mRNAs) in plants and animals (Bartel, 2004). miRNAs biogenesis is a multi-step process requiring several enzymes. First, in the nucleus, a primary transcript (pri-miRNA) is folded into a characteristic hairpin structure that is asymmetrically and specifically cut near the stem-loop by Drosha. Second, this newly generated miRNA (pre-miRNAs) is then transported to the cytoplasm, and further processed by Dicer to generate a heteroduplex (Lee et al., 2003). Finally, the double-strand duplex is unwound into two single strands, mature miRNA and miRNA*. The mature miRNA is usually responsible for silencing gene expression by mRNA cleavage, when the sequence is perfect complementarity with the target (Bartel, 2004), or by translation repression or RNA deadenylation, if complementarity is not perfect (Cuellar and McManus, 2005; Giraldez et al., 2006; Wu et al., 2006). The miRNA* was thought to have no regulatory activity; yet, recent evidence has shown that this molecule may also play important roles in gene regulation (Jagadeeswaran et al., 2010).

According to genomic location, miRNAs can be intergenic or non-intergenic (exonic or intronic) (Kim and Nam, 2006). Clusters of proximal miRNAs tend to be intergenic and are expressed as polycistronic, co-regulated units that contain their own promoters (Altuvia et al., 2005; Saini et al., 2007), whereas non-intergenic miRNAs are typically co-expressed with their host gene, although, instances of independent transcriptional regulation have also been reported (Tang and Maxwell, 2008; Bell et al., 2010).

Even though the biological functions of most animal miRNAs are little known, increasing evidence suggests that miRNAs play important roles in diverse physiological processes such as homeostasis (Reinhart et al., 2000), development (Lagos-Quintana et al., 2002), cancer pathogenesis (Calin et al., 2004), metabolism (Esau et al., 2006) and immune defense (Lu and Liston, 2009).

Recently, the role of miRNAs in both adaptive and innate immunity has attracted a great deal of interest (O'Connell et al., 2010). In mammals, the immune system consists of an innate response mediated via antimicrobial peptides (AMP) and an adaptive response mediated via antibodies. The adaptive immunity generates a large repertoire of antibodies by a mechanism involving somatic rearrangement of germ-line genes in response to specific antigens (Litman et al., 2010). The insects lack the adaptive response, but mount effective innate responses such as the synthesis of AMPs, phagocytosis, coagulation, encapsulation and melanization of pathogens (Lemaitre and Hoffmann, 2007). Interestingly, the innate immune response is partially conserved

Abbreviations: miRNA, microRNA; mRNA, messenger RNA; UTR, untranslated region; AMP, antimicrobial peptide; NF-κB, nuclear factor-kappa B; PRR, pattern recognition receptor; TLR, Toll-like receptor.

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between mammals and insects. Indeed, the *Drosophila* immune system has proven to be an extremely powerful model of the human response. For example, the mammalian Toll-like receptors (TLR) were identified by homology to *Drosophila* Toll (Medzhitov and Janeway, 1997) and the intracellular components of the Toll-signaling pathway show high similarities between flies and mammals. In *Drosophila,* the humoral response involves both, the localized melanization (Medzhitov and Janeway, 1997) and the production of AMPs in the fat-body, which is the analog of the mammalian liver (Lemaitre and Hoffmann, 2007), while the cellular response is governed by hemocytes, which phagocytose or encapsulate the microorganisms (Williams, 2007). AMP expression is mainly regulated by two signaling pathways: Toll and Imd (immune deficiency), showing similarities to the mammalian Toll-like receptor/interleukin-1 and tumor necrosis factor-α pathways, respectively (Lazzaro, 2008). The Toll pathway, which is a serine protease cascade, activates the immune response against fungal and Gram-positive bacterial challenge and consists of three different branches: two pattern recognition receptor (PRR)-dependent pathways and the virulence dependent "danger-signaling" pathway. The peptidoglycans of Gram-positive bacterial cell walls bind to the pattern recognition receptors PGRP-SA, PGRP-SD and GNBP1; while the β-1,3-glucans of fungal cell walls are recognized by GNBP3 receptor. The virulence factors secreted by fungi and bacteria induce the proteolytic cascade of the danger-signaling pathway (El Chamy et al., 2008). The Imd pathway involves a kinase cascade activated

Table 1
Immune-miRNAs of *Drosophila melanogaster*. *Drosophila* immune genes are identified by gene names and Flybase CG number. The putative miRNAs targeting immune genes are arranged in the third column following the next nomenclature: miR = mature form of miRNA; miR-X-1 and miR-X-2 = identical but come from different pre-miRNA; miR-X and miR-X* = miRNA with * is expressed at low levels relative to the miRNA in the opposite arm of the hairpin; miR-Xa and miR-Xb = very similar. Note that the same miRNA can appear related to several genes.

Gene name	Gene ID	miRNA
Potential miRNA targets of the Imd pathway		
PGRPLC	CG4432	bantam, miR-31a, miR-31b, miR-283, miR-1003
PGRPLE	CG8995	miR-33, miR-280
PGRPLF	CG4437	bantam, miR-1007, miR-1012
imd	CG5576	miR-11, miR-92b, miR-283, miR-306*, miR-312, miR-313
Fadd	CG12297	miR-33, miR-124, miR-1003
Tab2	CG7417	miR-2a, miR-2b, miR-2c, miR-6, miR-11, miR-13a, miR-13b, miR-289, miR-308, miR-1011
Takl	CGl8492	miR-5, miR-263a, miRNA-275
ird5	CG4201	bantam, miR-4, miR-31b, miR-79, miR-279, miR-286, miR-289, miR-315
key	CG16910	miR-3, miR-33, miR-306*, miR-309
Dredd	CG7486	miR-184, miR-284, miR-1013
Rudra	CG15678	miR-31a, miR-31b, miR-274, miR-276a
Dpt	CG12763	miR-306
Dro	CG10816	miR-184*
Potential miRNA targets of the Toll pathway		
GNBP3	CG5008	miR-8
PGRP-SD	CG7496	miR-33, miR-287, miR-1010
psh	CG6367	miR-2a, miR-6, miR-11, miR-13b, miR-308, miR-1011
ModSP	CG31217	miR-2b, miR-6, miR-9a, miR-9b, miR-11, miR-305, miR-316, miR-1010
Grass	CG5896	miR-5
SPE	CG16705	miR-11, miR-308
spz	CG6134	miR-284, miR-304, miR-1017
Spn1	CG9456	miR-275, miR-306, miR-313
Myd88	CG2078	miR-3, miR-219, miR-286, miR-318
pll	CG5974	miR-1, miR-303, miR-314
tub	CG10520	miR-12, miR-281
cact	CG5848	miR-210, miR-1016
dif	CG6794	miR-287
Mtk	CG8175	miR-34, miR-263a, miR-287
Potential miRNA targets of JNK pathway		
bsk	CG5680	miR-9a, miR-9b, miR-263a, miR-274
hep	CG4353	let-7, miR-92b, miR-310, miR-311, miR-313
Pvfl	CG7103	miR-8
Pvf2	CG13780	miR-8, miR-314
Pvf3	CG31629	let-7, miR-277, miR-304, miR-306, miR-317, miR-1016
Pvr	CG8222	miR-4, miR-79, miR-125, miR-263a, miR-314
Sulfl	CG6725	miR-14, miR-276*, miR-1012
puc	CG7850	miR-79, miR-276*, miR-1004
Jra	CG2275	miR-124, miR-184*
kay	CG33956	miR-2b, miR-13a, miR-13b, miR-92b, miR-184*
Potential miRNA targets of JAK/STAT pathway		
upd	CG5993	miR-1, miR-13a, miR-13b, miR-279, miR-286, miR-303, miR-306*, miR-308
dome	CG14226	miR-306
hop	CG1594	miR-306*
Potential miRNA targets of melanization pathway		
MP1	CG1102	miR-125, miR-287
MP2	CG3066	miR-2b, miR-13a, miR-281-2*
Spn27A	CG11331	miR-280
Potential miRNA targets of TNF pathway		
Eiger	CG12919	miR-275, miR-309

through the PGRP-LC and PGRP-LE receptors, which detect components of Gram-negative bacterial cell walls (Lemaitre and Hoffmann, 2007). Both the Toll and the Imd pathways culminate in the activation of NF-κB related transcription factors and the transcription genes such as those encoding the effector AMPs. In addition, JAK–STAT pathway counters viral infection (Dostert et al., 2005), whereas JNK cascade protects from Gram-negative bacteria (Bond and Foley, 2009).

Misregulation of the intensity and duration of the immune response, which is tightly regulated at multiple stages, can provoke cancer, chronic inflammatory disorders and developmental defects (Han and Ulevitch, 2005). Abnormal miRNA levels are likely one cause of these pathologies. Therefore, clarifying the complexity of the miRNA network involved in the immune system appears to be necessary for a better understanding of immunological disorders. Because of the aforementioned overlapping of their immune systems and because the vast majority of identified animal miRNAs are conserved across species (Ibanez-Ventoso et al., 2008), studying the *Drosophila* miRNAs could seed light into the understanding of the human immune system as well.

miRNA-mediated gene regulation requires sequence complementarity with the mRNA, therefore algorithms based on sequence similarity have been developed to define targets for miRNAs (Rajewsky, 2006). Yet, assigning a biological function to a miRNA based on its potential targets is difficult because (i) different transcripts may be regulated by the same miRNA and (ii) different miRNAs may regulate the same mRNA (Brennecke et al., 2005). Therefore, the identification of miRNAs involved in a particular process requires a more comprehensive method than that based on listing their potential targets.

In this study, we have defined a set of criteria to find a subset of seven miRNAs that may play an important role in the *Drosophila* immune system. The screening strategy used, which can be extended to other organisms and signaling pathways, is based on the identification of miRNA targets, their conservation across species and their expression profiles.

2. Material and methods

In order to identify putative miRNAs that target genes involved in *Drosophila* immune response, a three-step screening strategy was designed.

2.1. Data collection of immune-miRNA sequences

To assemble a set of *Drosophila* miRNA sequences, the MicroCosm Targets website (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) was used. This repository contains computationally predicted targets for the miRNAs in the miRBase release 16 (September 2010, http://www.mirbase.org/). From the 176 *Drosophila* miRNAs, only 93 have assigned putative targets in MicroCosm Targets (Supplementary File 1) and from those, only the ones targeting transcripts with known role in the immune response were selected for further analysis.

2.2. Sequence conservation analysis

To assess sequence conservation among *Drosophila melanogaster*, *Caernohabditis elegans* and *Homo sapiens*, two criteria described by Ibanez-Ventoso et al. (2008) were used: identity over seven continuous nucleotides at the 5′ end (1–10 nucleotides) and ⩾70% overall similarity over the full length. The sets of both, hairpin and mature miRNA sequences were aligned using CLUSTALW with default parameters (Thompson et al., 1994).

2.3. Determination of expression pattern

The genomic location of each miRNA with immune-related targets was obtained from miRBase. If in that specific location a gene is also present, called host gene, the expression profile of the corresponding miRNA could be inferred from the expression data of the gene. FlyAtlas provides a microarray-based atlas of gene expression in multiple tissues of *Drosophila* adult and larvae and

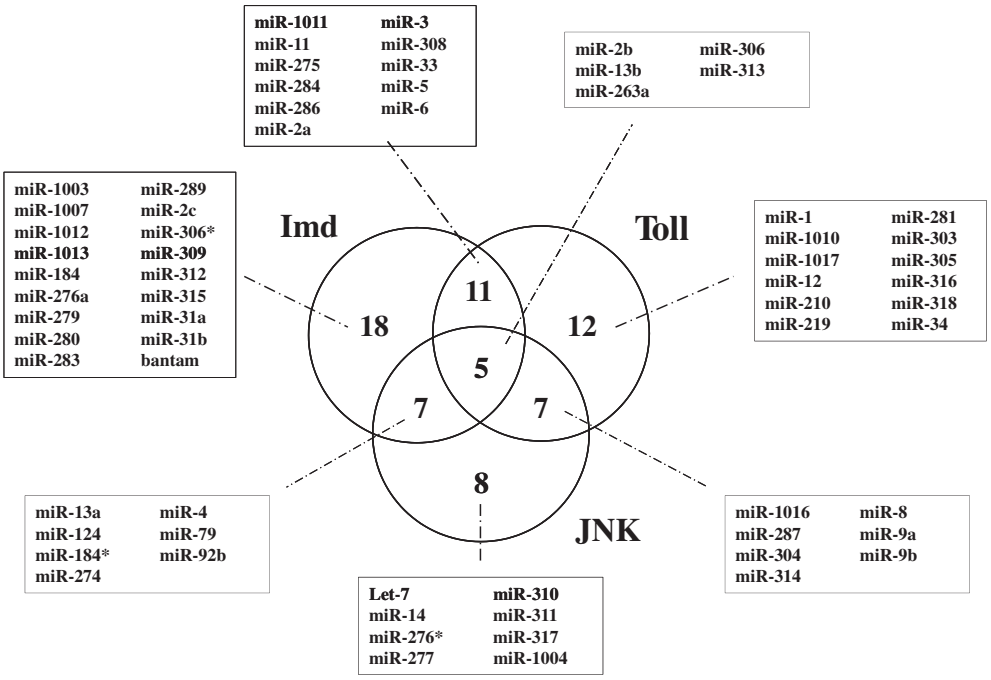

Fig. 1. Venn diagram of putative miRNAs targeting genes of Toll, Imd and JNK pathways in *Drosophila*. miRNAs that target *in silico* genes involved in Toll, Imd and JNK cascades are shown. While some miRNAs are restricted to one pathway (12 for Toll, 18 for Imd and 8 for JNK), others are common for two or three cascades. Toll and Imd share 11 miRNAs; Toll and JNK have seven miRNAs in common, whereas seven miRNAs are listed in JNK and Imd group. In addition, five miRNAs are putative regulators of genes involved in all three pathways.

gives a ranking of expression level for each gene in each tissue (http://flyatlas.org/). To assign the expression profile of each miRNA, the tissue with the highest expression level of its host gene was chosen.

3. Results and discussion

3.1. MicroRNAs in Drosophila immune system

Up to now 176 miRNAs have been described in *D. melanogaster*. Out of these, 73 are putative "immune-miRNAs", meaning that their potential target genes are components of Toll, Imd, melanization, JNK and JAK/STAT pathways (Table 1). Forty-four transcripts involved in these pathways show potential binding sites for miRNAs: 14 genes belonging to Toll; 13 genes to Imd; three genes to melanization; 10 genes to JNK; three genes to JAK–STAT and one gene to TNF (Table 1). Genes that do not appear in the table have no putative binding sites for miRNAs such as *GNBP1*, *PGRP-SA*, *necrotic*, *drosomycin* and *relish*. Since Toll, Imd and JNK cascades are the most described, we have focused on miRNAs that are likely associated to these three pathways. While some miRNAs only appear in one pathway, 12 miRNAs for Toll, 18 miRNAs for Imd and eight miRNAs for JNK, others are common for two or three pathways (Fig. 1). It is known that the same miRNA can target several transcripts belonging to different pathways (Brennecke et al., 2005). This redundancy in miRNA-mediated regulation suggests the existence of cross-talk between immune-related cascades, which has been demonstrated previously in flies (Lemaitre and Hoffmann, 2007) and large beetles (Kan et al., 2008). Indeed, in *Drosophila* 12,046 mRNA transcripts containing potential binding sites for miRNAs have been described *in silico*, suggesting that several transcripts are controlled by the same miRNA. Thus, a putative immune-miRNA might participate in the regulation of distinct biological processes.

3.2. Structure of immune-microRNA clusters

In *Drosophila*, over 35% of miRNAs form clusters, which are defined by default as a 10 kb genomic region. Thirty-six miRNAs out of the 73 immune miRNAs examined fall into clusters and share genomic location with other immune-related miRNAs, with the exception of mir-11, mir-34, mir-303 and mir-318. The remaining 37 immune-miRNAs are single transcripts. Some of the clusters are strongly conserved among animals, such as mir-100/let-7/mir-125, mir-277/mir-34, mir-275/mir-305 and mir-12/mir-283 (Supplementary File 2). In general, members of one cluster have arisen from a common pre-miRNA implying that they show a pattern of sequence conservation. However, miRNAs are especially subject to change, adaptation and functional diversification, which makes the identification of conservation patterns among members of one cluster difficult.

3.3. Conservation of miRNAs within clusters

The seed, a segment at 5′ end region of miRNAs, normally defines the conservation degree, and seeds are often similar throughout diverse species (Ruby et al., 2007). The arms of the hairpin precursors are less conserved than seeds, but are more conserved than the surrounding genomic sequence or the intervening loop sequence (Lai et al., 2003). In order to assess sequence similarity, immune-miRNAs of each cluster were aligned. Neither hairpin nor mature immune-miRNAs showed sequence conservation indicating that divergence is considerably high (data not shown). The lack of sequence conservation across immune-miRNAs might suggest functional specialization of each miRNA, which is derived from the necessity of adaptation to face fast-evolving pathogens.

3.4. Conservation of miRNAs across species

Based on the two criteria described in Section 2, sequence similarity between mature miRNAs of *Drosophila*, *C. elegans* and *H. sapiens* was studied. From the 73 immune-miRNAs presented in our study 37 are conserved between *Drosophila*, *C. elegans* and humans; 17 miRNAs between *Drosophila* and humans, six between *Drosophila* and *C. elegans* and 10 are specific for flies. Three miRNA sequences (miR-3, miR-14 and miR-318) are listed in both, *Drosophila*_human and *Drosophila_C. elegans* groups but not in the group formed by three species (Table 2). This happens since no conservation was found in *C. elegans* and *H. sapiens* sequences under the criteria previously mentioned. miR-287, miR-303, mir-317, miR-1011, miR-1012 and miR-1013 show sequence similarity of <70% overall with either nematodes or humans, so these miRNAs have been assigned as "not conserved".

In general, significant conservation of immune-miRNAs exists across flies, nematodes and humans. miRNAs that are conserved

Table 2
Conservation of immune-miRNAs across species. Thirty-seven out of 73 *D. melanogaster* immune-miRNAs share 5′ end identities and/or ⩾70% conservation over sequence with miRNAs both in nematodes and humans. Seventeen fly miRNAs show sequence conservation only with humans and six miRNAs are shared by flies and nematodes. In addition, 10 miRNAs appear to be restricted to *D. melanogaster*. In summary, flies and worms show similarity in 43 miRNAs, whereas flies and humans in 54 miRNAs. Note that miR-3, miR-14 and miR-318 are listed in both *Drosophila*_human and *Drosophila_C. elegans* groups but not in fly_nematode_human group. The reason is that *C. elegans* and *H. sapiens* miRNA sequences do not show sequence similarities under the criteria followed in this work.

dme-miRNA		Conservation
1	124	*Caernohabditis elegans* and *Homo sapiens*
2a	125	
2b	263a	
2c	277	
4	279	
6-1	281-1	
6-2	281-2	
6-3	281-2*	
8	286	
9a	304	
9b	306*	
11	308	
13a	310	
13b	311	
31a	312	
31b	313	
34	bantam	
79	let-7	
92b		
3	283	*Homo sapiens*
12	306	
14	314	
33	316	
184	318	
210	1003	
219	1010	
274	1016	
276a		
3	305	*Caernohabditis elegans*
14	318	
281	1007	
5	289	*Drosophila melanogaster*
184*	309	
275	315	
280	1004	
284	1017	

Table 3
Immune-miRNAs expressed in immune-related tissues and conserved in *D. melanogaster* and humans. Non-intergenic *Drosophila* immune-miRNAs identified using our screening strategy, which are conserved in flies and humans, are listed. Localization of these miRNAs within their host genes (intron or exon) is shown. Host genes are expressed in immune-related tissues according to information obtained from FlyAtlas website.

dme-miRNA	Host gene	Localization	Expression
mir-1003	Sense CG6695	Intron	Larval CNS, salivary gland, midgut, fat-body
mir-1016	Sense opa-1-like	Exon	Fat-body, hindgut
mir-12	Antisense CG32584	Intron	Testis, larval fat-body
mir-283	Antisense CG32584	Intron	Testis, larval fat-body
mir-304	Antisense CG32584	Intron	Testis, larval fat-body
mir-31b	Sense CG10962	Intron	Larval fat-body
mir-33	Sense Paps	Intron	Larval fat-body, salivary gland

among different species are likely to regulate the same biological function. This presumption agrees with the host–pathogen co-evolution idea, meaning that reciprocal genetic changes occur in interacting species owing to natural selection imposed by each on the other. Because the immune system is one of the fastest evolving systems, proteins, as well as regulatory elements such as miRNAs, involved in the immune pathways have to adapt rapidly to environmental changes. Transcription, translation and protein-folding impose many constraints, making evolution of protein-coding genes slower. However, the exceptional flexibility and versatility of miRNAs appear to be crucial features to evolve rapidly (Liu et al., 2008).

3.5. Spatial and temporal expression patterns of immune-miRNAs

From the 73 immune-miRNAs, 43 are intergenic and 30 are non-intergenic. Among the 30 non-intergenic miRNA, 29 are intronic and one is exonic (Table 3 and Supplementary File 3). Because, non-intergenic miRNAs usually show identical expression patterns to their host genes, expression profiles of host genes were checked. These genes are mainly expressed in 11 different tissues such as larval central nervous system (CNS), brain, salivary gland, thoracicoabdominal ganglion, larval fat-body, midgut, hindgut, tubule, eye, testis, ovary and male accessory gland. Interestingly, seven host genes out of 20 are most expressed in larval fat-body and/or in midgut/hindgut (Supplementary File 3), which are tissues related to the immune response in *Drosophila*. Seven miRNAs (miR-12, miR-31b, miR-33, miR-283, miR-304, miR-1003 and miR-1016) out of 10 that target host genes expressed in immune-related tissues are conserved in flies and humans (Table 3). These miRNAs are likely to play a role in the immune response in flies as well as in humans.

The determination of the transcription profile of intergenic miRNAs is difficult and identification of transcription factor/promoter interaction is crucial. The expression pattern of the 43 intergenic miRNAs described in this study is unknown, therefore their involvement in immune-related pathways cannot be ruled out. In fact, two intergenic miRNAs analyzed in this study, let-7 and miR-125, are known to regulate TLR-signaling in mammals. The let-7 family members control *TLR4* and *interleukin-6* gene expression, whereas miR-125 destabilizes *tumor necrosis factor* (*TNF*) cytokine mRNA by direct binding (O'Neill et al., 2011). Let-7 and miR-125, which are strongly conserved in *Drosophila* and humans, are clustered, suggesting that they are co-ordinately transcribed. Interestingly, in flies, the 3′UTR of *diptericin* gene, which encodes an antimicrobial peptide, contains a binding site for let-7; while *TNFα* is thought to be bound by miR-125 (Garbuzov and Tatar, 2010). The possible role of these two miRNAs in the *Drosophila* innate immune response strengthens the validity of our screening strategy.

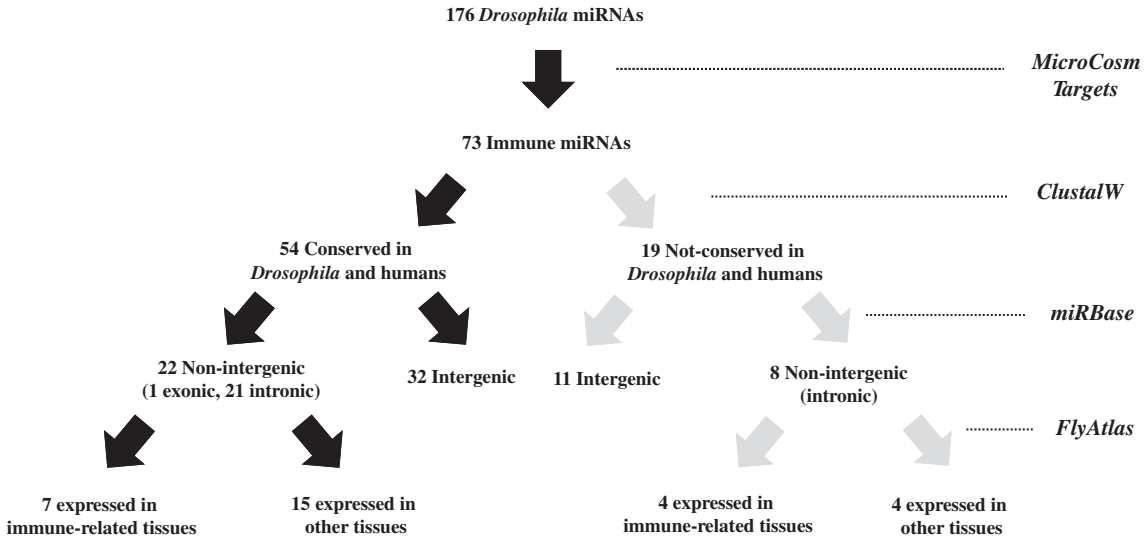

Fig. 2. Overview of the screening strategy. The screening strategy is based on three criteria to identify miRNAs involved in the immune system: (i) the identification of miRNAs targeting known immune-related genes (MicroCosm Targets); (ii) the sequence conservation of miRNAs across species (CLUSTALW), (iii) the determination of the expression profiles of host genes (FlyAtlas). Fifty-four miRNAs out of 73 immune-miRNAs are conserved in *Drosophila* and humans, while 19 miRNAs are not conserved. Twenty-two conserved miRNAs out of 54 are located within introns or exons of protein-coding genes and seven out of these 22 miRNAs are expressed in immune-related tissues (fat-body, midgut and hindgut). On the other hand, 19 immune-miRNAs are not conserved in flies and humans (those miRNAs conserved in *C. elegans* and *Drosophila* and, fly specific miRNAs are considered "not conserved"). Eight miRNAs out of these 19 not conserved miRNAs are non-intergenic and four miRNAs out of these miRNAs are expressed in immune-related genes.

3.6. Roles of miRNAs in the regulation of immune system

Following the three-step screening strategy we have obtained a set of seven immune-miRNAs. Components of the Imd and the Toll signaling pathways are putative targets of these miRNAs: *tub* for miR-12; *PGRP-LC*, *ird5* and *Rudra* for miR-31b; *PGRP-LE*, *PGRP-SD*, *FADD* and *key* for miR-33; *PGRP-LC* and *imd* for miR-283; *spz* and *Pvf3* for miR-304; *PGRP-LC* and *FADD* for miR-1003, and *cact* and *Pvf3* for miR-1016. Interestingly, the receptors *PGRP-LC*, *PGRP-SD* and *PGRP-LE* are putative targets of our candidates. These proteins are recognition molecules that detect pathogen-associated molecular patterns in microorganisms, initiating the innate immune response. Because *PGRP-LC* and *PGRP-SD* are components of the Imd and Toll signaling pathways, their expression levels are induced after bacterial infection with *E. coli* and *M. luteus*, respectively. On the contrary, the cytoplasmic *PGRP-LE* receptor does not show changes in transcription levels after these types of infection (De Gregorio et al., 2002). PGRP-LE recognizes intracellular pathogens such as *Listeria monocytogenes* and induces autophagy, a biological process independent of the humoral response (Yano et al., 2008). Regulation of these receptors appears to be essential to control the activation of the immune pathways, although no inhibitors of them are known yet in *Drosophila*. However, it has been shown that miRNAs are important controllers of TLR signaling in mammals. TLRs, which are the functional equivalent of *Drosophila* PRRs, have to be tightly regulated to avoid excessive inflammation and to allow for tissue repair and the return to homeostasis after infection and tissue injury. Several miRNAs are induced by TLR activation in innate immune cells, suggesting that these miRNAs regulate the strength, location and timing of TLR responses. However, functional data showing the exact effects of miRNAs on TLR signaling are still required (O'Neill et al., 2011).

To date, no immunological function has been assigned to the seven immune-miRNAs identified in this study. However, mir-31 has been reported to be related to colon, lung and endometrial cancers (Aprelikova et al., 2010; Wang et al., 2010; Xi et al., 2010) and miR-33 deregulation represents a new mechanism of p53 inhibition and neoplastic proliferation in human cancer (Herrera-Merchan et al., 2010).

4. Conclusion

We have described a screening strategy to identify miRNAs involved in *Drosophila* immune system that is based on (i) the identification of miRNAs that potentially target known immune-related genes, (ii) the sequence conservation of those miRNAs across different species and (iii) the determination of their expression profiles (Fig. 2). This strategy is applicable to distinct studies that aim to identify miRNAs involved in any pathway of interest.

In general, the assignment of biological functions of miRNAs is a great challenge due to the difficulty in identifying their target genes. Deep-sequencing technologies have considerably raised the rate of novel miRNA discovery and can help establishing miRNA/pathology relationships. However, massive sequencing strategies are not always applicable owing to the cost. Consequently, a different way to deal with the functional characterization of miRNAs is necessary. The main goal of our screening procedure is helping researchers delimit the starting set of miRNAs that possibly act in the signaling pathway of interest. Thus, the number of miRNAs to be studied is lower, facilitating the subsequent experimental approaches.

In the future miRNAs might be used as diagnostic, prognostic and therapeutic tools. miRNA therapies could involve (i) administrating a specific miRNA to downregulate specific target genes, for example, in some cancer cell lines, or (ii) blocking certain miRNAs using "antagomirs", chemically engineered anti-miRNA oligonucleotides, to increase expression of target genes. However, in either case, the immediate priority lies in identifying miRNAs that regulate important signaling pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2011.03.034.

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