

INSECT MOLECULAR BIOLOGY AND BIOCHEMISTRY



Edited by LAWRENCE I. GILBERT



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Amsterdam • Boston • Heidelberg • London • New York • Oxford Paris • San Diego • San Francisco • Singapore • Sydney • Tokyo Academic Press is an imprint of Elsevier



Academic Press is an imprint of Elsevier 32 Jamestown Road, London NW1 7BY, UK 225 Wyman Street, Waltham, MA 02451, USA 525 B Street, Suite 1800, San Diego, CA 92101-4495, USA

First edition 2012

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British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data

A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-384747-8

For information on all Academic Press publications visit our website at elsevierdirect.com

Typeset by TNQ Books and Journals Pvt Ltd. www.tnq.co.in

Printed and bound in China

 $10\ 11\ 12\ 13\ 14\ 15\ 10\ 9\ 8\ 7\ 6\ 5\ 4\ 3\ 2\ 1$

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PREFACE

In 2005 the seven-volume series "Comprehensive Molecular Insect Science" appeared and summarized the research in many fields of insect research, including one volume on Biochemistry and Molecular Biology. That volume covered many, but not all, fields, and the newest references were from 2004, with many chapters having 2003 references as the latest in a particular field. The series did very well and chapters were cited quite frequently, although, because of the price and the inability to purchase single volumes, the set was purchased mainly by libraries. In 2010 I was approached by Academic Press to think about bringing two major fields up to date with volumes that could be purchased singly, and would therefore be available to faculty members, scientists in industry and government, postdoctoral researchers, and interested graduate students. I chose *Insect Molecular Biology and Biochemistry* for one volume because of the remarkable advances that have been made in those fields in the past half dozen years.

With the help of outside advisors in these fields, we decided to revise 10 chapters from the series and select five more chapters to bring the volume in line with recent advances. Of these five new chapters, two, by Subba Palli and by Xavier Belles and colleagues, are concerned with techniques and very special molecular mechanisms that influence greatly the ability of the insect to control its development and homeostasis. Another chapter, by Park and Lee, summarizes in a sophisticated but very readable way the immunology of insects, a field that has exploded in the past six years and which was noticeably absent from the Comprehensive series. The other two new chapters are by Yong Zhang and Pat Emery, who deal with circadian rhythms and behavior at the molecular genetic level, and by Philip Jensen, who reviews the role of TGF- β in insect development, again mainly at the molecular genetic level. In most cases the main protagonist is *Drosophila melanogaster*, but where information is available representative insects from other orders are discussed in depth. The 10 updated chapters have been revised with care, and in several cases completely rewritten. The authors are leaders in their research fields, and have worked hard to contribute chapters that they are proud of.

I was mildly surprised that, almost without exception, authors who I invited to contribute to this volume accepted the invitation, and I am as proud of this volume as any of the other 26 volumes I have edited in the past half-century. This volume is splendid, and will be of great help to senior and beginning researchers in the fields covered.

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2 Insect MicroRNAs: From Molecular Mechanisms to Biological Roles

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Summary

MicroRNAs (miRNAs) are endogenous, *ca.* 22-nucleotide, single-strand, non-coding RNAs that regulate gene expression by acting post-transcriptionally through basepairing between the so called "seed" sequence of the miRNA (nucleotides 2–8 at its 5' end) and its complementary seed match sequence present in the 3' untranslated region of the target mRNA. Since the discovery of the first miRNAs in the 1990s, a remarkable diversity of miRNAs has been reported in various organisms, including insects, plants, viruses, and vertebrates. Moreover, computational methods have been developed to find new miRNAs as well as mRNA targets. In insects, most miRNAs are involved in modulating a precise dosage of regulatory proteins, thus fine-tuning biological processes like cell proliferation, apoptosis and growth, oogenesis and embryogenesis, nervous system and muscle differentiation, metamorphosis and other morphogenetic processes, and response to biological stress. The miRNA field is still developing, and many questions remain to be solved. Technologies to determine new miRNAs and miRNA targets still need refinement. Further studies are also needed to elucidate the mechanisms regulating miRNA expression, to validate the miRNA targets *in vivo*, and to establish the complex networks that connect miRNAs, mRNAs, and proteins, and that govern the development and function of cells and tissues.

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2.1. Introduction: The Big World of Small RNAs

Step by step, some of the old paradigms of molecular biology have been falling away. The most significant of these is the central dogma that "one gene equals one protein." It still holds true that most information flows from DNA to proteins through intermediate RNA molecules, but today it is well known that the transcriptome is much more complex and diverse than the genome, thanks to the interplay of a variety of mechanisms. The most thoroughly studied is alternative splicing; that is, the formation of diverse mRNAs through differential splicing of the same RNA precursor, which gives rise to proteins with distinct features. Another factor accounting for transcriptome diversity in quantitative terms and in time and space is the occurrence of transcription factors, sequence-specific DNA-binding factors that usually bind to the promoter region of target genes, thereby activating or repressing their transcription. However, to understand thoroughly the dynamics of the proteome, we have to account for the unknown mechanisms other than simply protein-coding genes and transcription factors. At least, non-coding RNAs (ncRNAs) must also be taken into account in order to have a more complete picture of what is really happening in genomic regulation.

ncRNAs form a heterogeneous group of RNA molecules that are classified into three categories according to their length and function. They range in length from 18 to 25 nucleotides for the group of very small RNAs, which includes short interfering RNAs (siRNAs) and microRNAs (miRNAs); from 20 to 200 nucleotides for the group of small RNAs, which usually play the role of transcriptional



Figure 1 Types of non-coding RNAs (ncRNAs) classified according to their length and functions: very small RNAs – microRNAs and small interfering RNAs (siRNAs); small RNAs; and medium and large RNAs. The corresponding established functions for each type are also indicated. snoRNAs, small nucleolar RNAs; smRNAs, small modulatory RNAs; piRNAs, Piwi-interacting RNAs. Data from Costa (2007).

and translational regulators; and, for the group of medium and large RNAs, up to (and even beyond) 10,000 nucleotides, which are involved in other processes, as detailed in **Figure 1** (Costa, 2007). This chapter deals with the very small RNAs, and, more specifically, with miRNAs.

The history of siRNAs and miRNAs began in the late 1980s, when Jorgensen and colleagues were studying the role of chalcone synthase in the biosynthetic pathway of anthocianin in plants. Anthocianin gives a violet color to petunias, and Jorgensen's team overexpressed chalcone synthase in search of petunias with a deeper violet color. However, they unexpectedly obtained whitish flowers because the expression of chalcone synthase in these transgenic whitish petunias was some 50 times lower than in the wild type, thus suggesting that transgenic chalcone synthase had suppressed the endogenous gene (Jorgensen, 1990). Three years later, but in the field of developmental biology and working on the nematode Caenorhabditis elegans, Lee and colleagues (1993) discovered two lin-4 transcripts, where the smaller, with ca. 21 nucleotides, was complementary to seven repeated sequences in the 3' UTR of the mRNA of the heterochronic gene lin-14, which had been identified two years earlier.

These two disparate studies converged in 1998, when Fire and colleagues (1998), also working in C. elegans, discovered that the administration of a double-stranded RNA (dsRNA) with a strand complementary to a fragment of an endogenous mRNA can block this mRNA. This phenomenon is now known as RNA interference (RNAi), and its action is mediated by siRNAs of ca. 22 nucleotides that derive from dsRNA (Belles, 2010). A year later, while studying post-transcriptional gene silencing as a mechanism of antiviral defense, Hamilton and Baulcombe (1999) noticed the occurrence of antisense viral RNA of ca. 25 nucleotides in virus-infected plants. Hamilton and Baulcombe observed that these small RNAs were long enough to convey sequence specificity, and pointed out that they might be key determinants of the gene silencing phenomenon. Further contributions showed that dsRNA-induced mRNA degradation was always mediated by RNAs of 21-23 nucleotides, thus leading researchers to investigate the endogenous source of these small RNAs. Finally, in 2001, three groups working independently (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) described miRNAs as a novel family of small (ca. 22 nucleotides) endogenous RNAs that is diverse in sequence and temporal expression, evolutionarily widespread, and involved in regulating gene expression.

2.1.1. RNAi and siRNAs

The discovery of RNAi in *C. elegans* (Fire *et al.*, 1998) was later extended to other animal groups, namely insects, and the basic mechanisms involved in their action on mRNAs were unveiled step by step in a few years. The biochemical

machinery and the effects of RNAi in insects have been the subject of a recent review (Belles, 2010), and we will not deal with them in detail here. In essence, when a long and exogenous dsRNA is delivered to the insect, it is cleaved by the enzyme Dicer-2 into siRNA duplexes of *ca.* 22 nucleotides. These siRNAs then unwind, and singlestrand siRNAs bind to Argonaute-2 protein (Ago-2) and assemble into the so-called RNA-induced silencing complex (RISC). The RISC, guided by the siRNA, couples to the target mRNA and degrades it (Belles, 2010). Indeed, the mechanisms of generation and the action of siRNAs are very similar to those of miRNAs (**Figure 2**), with the latter detailed in the following sections.

The advent of RNAi represented a new paradigm in insect functional genomics, because it opened the door for studying non-drosophilid species - that is, species that cannot be genetically transformed, at least not very easily. RNAi experiments are relatively simple, consisting of conveying a dsRNA with a strand complementary to a fragment of the target mRNA to the animal or to cells incubated in vitro. After assessing that target mRNA levels have lowered, the study of the phenotype unveils the functions associated with the target mRNA. Experiments can be carried out in vitro, where the easiest system involves the incubation of cells with dsRNA added to the medium and then studying the cell behavior; and in vivo, where the most straightforward approach consists in delivering the dsRNA to the chosen insect stage (from egg to adult) of the experimental specimen and then examining the resulting phenotype. The approaches in vivo have afforded the most spectacular results on insect functional genomics (Belles, 2010).

Kennerdell and Carthew (1998) were the first to use RNAi in vivo in insects, studying the genes frizzled and frizzled 2 in the fly Drosophila melanogaster. A year later, Brown and colleagues (1999) carried out functional studies of Hox genes in the flour beetle Tribolium castaneum, and the following year used RNAi for the first time in a hemimetabolan species, the milkweed bug Oncopeltus fasciatus, on which Hughes and Kaufman (2000) studied Hox gene functions as well. In 2006, RNAi in vivo was used for the first time on a phyllogenetically very basal insect, the German cockroach Blattella germanica (Ciudad et al., 2006; Martin et al., 2006), which has been shown to be one of the species most sensitive to RNAi. In the past few years an explosion of papers has truly changed the landscape of reverse functional genetics in insects, and has unveiled many gene functions, from development to reproduction, including behavior, coloration, resistance to biological stress, polyphenism, and many others (Belles, 2010).

2.1.2. miRNAs

miRNAs are endogenous, *ca.* 22-nucleotide, single-strand, non-coding RNAs that regulate gene expression on the

post-transcriptional level through base-pairing between the seed sequence of the miRNA and its complementary seed match sequence that is present in the 3' untranslated region (UTR) of the target mRNA.

The first miRNA, lin-4, was discovered in a screen for genes required for post-embryonic development in the nematode *C. elegans* (Lee *et al.*, 1993; Ambros and Horvitz, 1984). The identification of the *lin-4* locus and its regulatory mechanism through the 3' UTR of *lin-14* mRNA was an interesting finding, although at that time it was almost considered to be a genetic oddity. However, the discovery of another miRNA, let-7, initially in *C. elegans* (Reinhart *et al.*, 2000) and later in various bilaterian species (Pasquinelli *et al.*, 2000), confirmed that, in the case of lin-4 and lin-14, it was not an oddity at all, but rather a new and fundamental layer of the mechanisms regulating gene expression (Lai *et al.*, 2003; Neilson and Sharp, 2008).

The following sections will deal exclusively with miRNAs.

2.2. Biogenesis of miRNAs

miRNAs undergo molecular processing before becoming mature and ready to play their functional role. The pathway of miRNA biogenesis has many commonalities with that of siRNAs, but it is distinct in a number of ways (**Figure 2**). miRNAs are first transcribed as part of a longer primary transcript (pri-miRNA), which folds, forming hairpin structures that correspond to miRNA precursors (pre-miRNAs). pri-miRNAs are then processed in the nucleus and transported to the cytoplasm, where they undergo final maturation (**Figure 2**).

2.2.1. miRNA Processing in the Nucleus

Most miRNA genes are transcribed by RNA polymerase II into pri-miRNA, although in some cases the transcription is mediated by RNA polymerase III (Lee *et al.*, 2004a; Borchert *et al.*, 2006). Usually, pri-miRNAs are several kilobases long, contain local stem-loop structures,



Figure 2 Biogenesis of miRNA and siRNA. miRNA gene is transcribed by RNA Pol II/III into a primary transcript (pri-miRNA) that is processed by Drosha/Pasha and exported to the cytoplasm by Exportin-5. In the cytoplasm, the precursor (pre-miRNA) undergoes the final step of maturation and is cleaved by Dicer-1/Loquacious into an miRNA duplex. After the miRNA duplex unwinds, the mature miRNA is maintained with Argonaute-1 protein (Ago-1) forming a RISC which will be coupled to the target mRNA and will degrade, destabilize, or translationally inhibit it, whereas the miRNA' is released and degraded. On the other hand, siRNA is formed when a long and exogenous double-strand RNA (dsRNA) is cleaved by Dicer-2/R2D2 into an siRNA duplex. Likewise with the miRNA pathway, the siRNA duplex is unwound and single-strand siRNAs are maintain with Argonaute-2 protein (Ago-2) forming a RISC which will recognize the target mRNA and degrade it.

and are polyadenylated and capped, as in current mRNAs (Cai et al., 2004; Lee et al., 2004a), although the cap and the poly(A) tail are removed during miRNA processing. miRNA genes can form clusters in the genome (Behura, 2007), or can be found isolated within an intronic region of protein-coding genes, or in introns and exons of non-coding RNAs (Rodriguez et al., 2004). Moreover, pri-miRNAs can be polycistronic, thus carrying the information of more than one miRNA. In insects, the group of miR-100, let-7, and miR-125 constitutes the best studied example of polycistronic pri-miRNA. The organization of this pri-miRNA is well conserved in many species of insects, and even in vertebrates (Figure 3), although the spacer regions between miRNA precursor sequences can vary considerably in structure and length. For example, the distance between the precursor of miR-100 and that of let-7 varies from ca. 100 bp in T. castaneum to ca. 3.9 kb in Anopheles gambiae, whereas the distance between the precursor of let-7 and that of miR-125 varies within the range of 250–450 bp (Figure 3) (Behura, 2007).

pri-miRNA processing into *ca*. 70- to 80-nucleotide pre-miRNAs takes place exclusively in the nucleus by the action of the microprocessor, a protein complex of *ca*. 500 kDa, which in *D. melanogaster* is composed by the RNase III enzyme Drosha and its partner, Pasha (Figure 2) (Denli et al., 2004). In general, insects possess a single pasha gene copy, except in the pea aphid Acyrthosiphon pisum, where four pasha-like genes have been recently reported (Jaubert-Possamai et al., 2010). Pasha protein (also known as DGCR8 in vertebrates) contains two double-stranded RNA-binding domains; it plays an essential role in miRNA processing by recognizing the substrate pri-miRNA and by determining the precise cleavage site, whereas Drosha actually cleaves the primiRNA (Denli et al., 2004). The two RNase domains of Drosha cleave the 5' and 3' arms of the pri-miRNA 11 base-pairs away from the single-stranded RNA/doublestranded RNA junction at the base of the hairpin stem (Figure 4) (Han et al., 2004); of note, a single nucleotide variation in an miRNA precursor stem can block Drosha processing (Duan et al., 2007).

Generally, cleavage of the pri-miRNA by Drosha occurs in an unspliced intronic region before mRNA splicing catalysis (Kim and Kim, 2007). However, some miRNA genes are located within intronic regions which themselves form a hairpin structure. In this special case, the action of Drosha is bypassed during pri-miRNA processing. After splicing of its host mRNA, the miRNA is



Figure 3 Organization of the primary transcript of miR-100, let-7, and miR-125 cluster in different insect species and the zebrafish. Numbers inside the boxes correspond to the length in base-pairs. The sequences were obtained from the miRBase (http://www.mirbase.org).



Figure 4 Precursor of miRNA let-7. In one of the arms of the stem-loop of the hairpin the mature miRNA sequence resides (in red); in another is the miRNA* sequence (in blue). The sites of cleavage for both enzymes Drosha and Dicer-1 are shown as purple and green lines, respectively. This *Culex quinquefasciatus* sequence was obtained in miRBase (http://www.mirbase.org/).

released from the intron, then exported from the nucleus to the cytoplasm, and is finally cleaved by Dicer (see below). This class of miRNA, called mirtrons, has been described in flies, nematodes, and mammals (Berezikov *et al.*, 2007; Okamura *et al.*, 2007; Ruby *et al.*, 2007a).

2.2.2. Pre-miRNA Transport from the Nucleus to the Cytoplasm

Once the pri-miRNA is processed in the nucleus, the resulting pre-miRNAs are exported to the cytoplasm by Exportin5 (EXP5) (Figure 2), which is a member of the nuclear transport receptor family, in complex with the cofactor Ran-GTP (Yi et al., 2003; Kim, 2004). EXP5 can recognize double-stranded RNA stems longer than 14 base-pairs along with a short 3' overhang (1–8 nucleotides), which ensures the export of only those pre-miRNAs correctly processed (Lund et al., 2004). Using RNAi, a number of authors have demonstrated the role of EXP5 in the nucleocytoplasmic transport of pre-miRNA. Knockdown of EXP5 mRNA decreases the levels of mature miRNAs, but does not lead to an increase of pre-miRNA levels in the nucleus, which suggests that protecting pre-miRNA from digestion in the nucleus is another important role of EXP5 (Yi et al., 2003; Lund et al., 2004).

2.2.3. miRNA Maturation by Dicer

In the cytoplasm, pre-miRNAs are cleaved by Dicer near the terminal loop (Figure 4), thus resulting in the release of a ca. 22-nucleotide miRNA duplex with two nucleotides protruding as overhangs at each 3'-end. Dicer is an ATP-dependent multidomain enzyme of the RNase family involved in the cleavage of small double-stranded RNAs. It was identified for the first time in D. melanogaster (Bernstein et al., 2001), and two Dicer homologs were later found in this fly, Dicer-1 and Dicer-2, which, in general, are involved in the miRNA and siRNA pathways, respectively (Lee et al., 2004b). RNAi of Dicer-1 in the last instar nymph of the cockroach B. germanica inhibits the formation of mature miRNAs and impairs the metamorphic process (Gomez-Orte and Belles, 2009), which confirms not only the role of Dicer-1 in miRNA biogenesis in a phyllogenetically basal insect, but also that of miRNAs in hemimetabolan metamorphosis (see below).

D. melanogaster Dicer-1 interacts with the protein Loquacious (also known as R3D1), which contains three double-stranded RNA-binding domains for pre-miRNA processing. Depletion of Loquacious results in pre-miRNA accumulation in *Drosophila* S2 cells, and immuno-affinity purification experiments revealed that Loquacious locates in a functional pre-miRNA processing complex along with Dicer-1, and stimulates the specific pre-miRNA processing activity (Saito *et al.*, 2005). Of note, both arms of the pre-miRNA stem loop structures are imperfectly paired, containing G:U wobble pairs and single nucleotide insertions (**Figure 4**). The imperfect base-pairing causes differences in thermodynamic properties and makes one strand of the duplex less stably paired at its 5' end. Generally, the strand with the lowest thermodynamic stability becomes the mature miRNA (guide strand), whereas the other strand (miRNA* or passenger strand) is degraded.

2.2.4. Regulation of miRNA Biogenesis and Stability

As a general principle, given that most of the miRNA genes are transcribed by RNA Polymerase II, the usual transcription factors associated with Pol II will influence their transcriptional control. A more specific modality of miRNA regulation is the process known as editing, which consists in a post-transcriptional change of RNA sequences caused by deamination of adenosine (A) to inosine (I), thus resulting in alterations in the base-pairing of the transcript. pri-miRNA transcripts modified by ADAR (adenosine deaminase acting on RNAs) have their biogenesis altered in downstream steps. These modifications of the pri-miRNA sequences may block the cleavages by Drosha and Dicer during miRNA maturation; moreover, edited mature miR-NAs can recognize other target mRNAs (Kawahara et al., 2007). Therefore, editing is a remarkable regulator of biogenesis, and in addition increases miRNA structural diversity and further extends the diversity of miRNA targets.

Regulation of the miRNA biogenesis pathway also involves feedback mechanisms, like the interplay of Drosha and Pasha, which regulate each other in a circuit of negative feedback. Drosha acts by cleaving a hairpin located in the 5' UTR of Pasha mRNA. Excess of Drosha decreases Pasha mRNA levels, whereas a reduction of Drosha elicits the reverse effect (Kadener et al., 2009b). Another example of feedback regulation involves human Dicer and the miRNA let-7, wherein Dicer is targeted by let-7 in sites within its coding region (Forman et al., 2008), or the reciprocal regulation showed by let-7 and the RNA-binding protein Lin-28, where let-7 suppresses Lin-28 protein synthesis whereas Lin-28 blocks let-7 maturation. Lin-28 is capable of blocking the cleavages mediated by both Drosha and Dicer; indeed, recombinant Lin-28 can block pri-miRNA processing, whereas knockdown of Lin-28 facilitates the expression of mature let-7 (Viswanathan et al., 2008). Lin-28 acts by inducing uridylation of the let-7 precursor at its 3' end, which elicits the degradation of the uridylated pre-let-7 because Dicer fails to process hairpin RNA structures with long 3' extensions (Heo et al., 2008).

Unlike 3' uridylation, 3' adenylation may have a stabilizing effect on miRNAs, at least in mammals. For example, a variant of miR-122 possesses a 3'-terminal adenosine that is added by cytoplasmic poly(A) polymerase GLD-2 after unwinding of the miR-122/miR-122* duplex, and this 3' adenylation appears to prevent shortening, thus stabilizing the miRNA (Katoh *et al.*, 2009). Apparently, adenylation and uridylation are two competing processes; it is interesting, in this sense, that addition of adenine residues in some small RNAs can prevent urydilation (Chen *et al.*, 2000).

2.3. Mechanism of Action of miRNAs

The functional role of a miRNA is ultimately characterized by its effects on the expression of target genes. Currently, the regulatory mechanisms involving miRNAs are related to mRNA cleavage or translational repression by binding to complementary sites usually located on the 3' UTR region of the mRNA (Carrington and Ambros, 2003; Lai, 2003; Ambros, 2004; Bartel, 2004). In contrast to the inhibitory effects, miRNAs can also stimulate the expression of target genes by upregulation of translation (Vasudevan et al., 2007; Orom et al., 2008). Moreover, miRNAs can also control cell fate by binding to heterogeneous ribonucleoproteins and lifting the translational repression of their target mRNAs; in this way, miRNAs act through a sort of decoy activity that interferes with the function of regulatory proteins (Beitzinger and Meister, 2010; Eiring et al., 2010). The present section, however, will emphasize the more widespread mechanisms, leading to mRNA translational repression, which start when the miRNA binds to Ago-1 protein and with the assembly of the RISC (Figure 2).

2.3.1. Argonaute Loading

The Argonaute (Ago) family can be divided into two subfamilies: the Piwi subfamily and the Ago subfamily. Piwi proteins are involved in transposon silencing, and are especially abundant in germ-line cells. Ago-subfamily proteins play key roles in post-transcriptional gene regulation by interacting with siRNAs (see above) and miRNAs, as detailed below.

After Dicer-1-mediated cleavage, the miRNA duplex binds to an Ago-1 protein in the RISC. To form an active RISC, the miRNA duplex has to unwind because only the mature miRNA binds to the Ago-1 protein, whereas the miRNA* is released. In human cells, miRNAs with a high degree of base-pairing in their pre-miRNA hairpin stem are initially processed by Ago-2, which cleaves the 3' arm of the hairpin (that is, the miRNA* strand) in the middle, thus generating a nicked hairpin (Diederichs and Haber, 2007). In this case, Ago-2 acts before Dicer-1-mediated cleavage and facilitates miRNA duplex dissociation, the removal of nicked strand, and the activation of RISC. These findings elucidated the crucial role of Ago proteins not only during RISC formation, but also in relation to the mechanism that determines which of the two strands will become the survivor mature miRNA.

Identification of the target mRNA by the RISC is based on the complementarity between the mature miRNA and the target mRNA site, and the degree of complementarity determines whether the target mRNA is degraded, destabilized, or translationally inhibited. Binding to Ago-1 greatly enhances miRNA stability, and although little is known about the half-life of individual miRNAs, it is clear that Ago-1 is a limiting factor for endogenous miRNA accumulation due to its protective function.

In D. melanogaster, miRNA* strands may accumulate bound to Ago-2, a protein initially thought to act exclusively in the siRNA pathway. Whether miRNA* binds to Ago-1 or to Ago-2 depends on the miRNA duplex structure, thermodynamic stability, and the identity of first 5'-end nucleotide - i.e., miRNA sequences beginning with cystidine will bind to Ago-2, whereas those beginning with uridine will bind to Ago-1 (Ghildiyal et al., 2010). A number of observations indicate that some miRNA* plays a role in the regulation of gene expression. These observations include that: (1) miRNA* 5' ends are more defined than their 3' ends, thus suggesting that there is a seed region involved in regulatory functions (Ruby et al., 2007b; Okamura et al., 2008; Seitz et al., 2008); (2) many miRNA* sequences are evolutionarily conserved (Okamura et al., 2008); (3) in D. melanogaster (Ruby et al., 2007b) and in the basal insect B. germanica (Cristino et al., 2011), tissue concentration of some miRNA* is higher than that of the corresponding miRNA partner.

2.3.2. Repression of Protein Translation

The RISC is the key element that regulates gene expression by repressing protein translation. The first step after RISC formation is the recognition of the target mRNA, mainly through the seed sequence. A number of studies have demonstrated the importance not only of the seed, but also of the whole 5' region of the miRNA during the interaction with the target mRNA. According to Brennecke and colleagues (2005), there are two categories of miRNA target sites in mRNAs. The first is called the "5' dominant site," and occurs when there is a near perfect base-pairing in the 5' end of the miRNA; this category can be subdivided into "canonical" (when both 5' and 3' ends have strong base-pairing with the miRNA site) and "seed" (when only the 5' region presents consistent basepairing). The second category is called "3' compensatory," and occurs when base-pairing between the miRNA seed sequence and its corresponding sequence in the target mRNA is weak, and thus a stronger base-pairing in the 3' region exerts a sort of "compensating" effect.

Initial experiments in *C. elegans* showed that the mi RNAs lin-4 and let-7 repress their respective target mRNAs through interactions with miRNA sites in the 3' UTR. Subsequently, many other cases of miRNA binding sites in the 3' UTR of mRNAs were reported, leading to the presumption that this was a general rule. However, recent findings have revealed that miRNAs can repress mRNAs through sites located in the open reading frame (ORF) or in the 5' UTR (Lee *et al.*, 2009).

The action of RISC on target mRNAs may proceed through different mechanisms. One of them involves postinitiation repression, as shown by experiments carried out in *C. elegans* where lin-4 inhibits the translation of lin-14 mRNA without reducing the mRNA levels and without affecting the shifting of polysomes, thus suggesting that the inhibition of mRNA translation occurs at the elongation step (Wightman *et al.*, 1993; Olsen and Ambros, 1999; Lee *et al.*, 2003). Other details accounting for this mechanism of action have been reported, and a model has been proposed describing the inhibition of ribosome elongation, the induction of ribosome drop-off, and the facilitation of nascent polypeptides proteolysis (Fabian *et al.*, 2009).

The second mechanism of RISC action is the acceleration of target mRNA destabilization, involving: (1) decapping of the m(7)G cap structure in the 5' end; and/or (2) deadenylation of poly A tail during the initial step of translation (Humphreys *et al.*, 2005). A number of reports using different experimental models have supported this second mechanism; for example, in zebrafish embryos and mammalian cells, miRNAs in the RISC accelerate mRNA deadenylation, which leads to fast mRNA decay (**Figure 5**) (Giraldez *et al.*, 2006; Wu *et al.*, 2006). In *Drosophila* cells both deadenylation and decapping require GW182 protein, CCR4: NOT deadenylase, and the DCP1: DCP2 decapping complexes. Depletion of GW182 in *Drosophila* cells leads to alteration of mRNA expression levels. However, in Ago-1depleted cells, GW182 can still silence the expression of target mRNAs, thus indicating that GW182 acts downstream of Ago-1, and that it is a key component of the miRNA pathway (Behm-Ansmant *et al.*, 2006a).

2.3.3. Processing Bodies and mRNA Storage

In many cases, the last step of miRNA action involves the processing bodies (P-bodies), which are discrete cytoplasmic aggregates that contain enzymes associated to mRNA decay, such as CCR4:NOT complex (deadenylase), DCP1: DCP2 complex (decapping), RCK/p54, and eIF4ET (general translational repressors). The aforementioned GW182 is additionally required for P-body integrity. Apparently, P-bodies are the place where RISC delivers its target mRNA to be degraded or to be stored (Figure 5). In human cells, for example, miR-122-repressed mRNAs that are maintained in P-bodies can be released from them under stress conditions, and subsequently be recruited by polysomes (Bhattacharyya et al., 2006). Behm-Ansmant and colleagues (2006b) have proposed a model where RISC binds to target mRNA through interactions with miRNA and Ago-1, and recruits GW182, which labels the



Figure 5 Once the miRISC is formed, the target mRNA can be taken to a special region of cytoplasm known as the P-body, where it will be degraded after decapping and deadenylation, or maintained in the P-body until released from it and recruited to polysomes.

transcript as a target for decay via deadenylation and decapping. Ago-1 and Ago-2 proteins have also been detected in P-bodies (Liu *et al.*, 2005), thus suggesting that both siRNA and miRNA pathways may end in these structures. Nevertheless, this does not mean that P-bodies are crucial for the functioning of these pathways, given that disruption of P-bodies after depletion of Lsm1, which is a key component of them, elicits a dispersion of Ago proteins into the cytoplasm, but does not affect siRNA and miRNA pathways (Chu and Rana, 2006).

2.4. Identification of miRNAs in Insects

Since the discovery of lin-4 and let-7 in the nematode C. elegans, a remarkable diversity of miRNAs has been reported in the genomes of various organisms, including insects, plants, viruses, and vertebrates (http:// www.mirbase.org). In insects, research on miRNAs was initially limited to D. melanogaster, but the availability of sequenced genomes from different species, as well as the development of new bioinformatic tools, has allowed the performance of systematic predictions of miRNAs in silico. Accordingly, computational methods based on the evolutionary conservation of genomic sequences and their ability to fold into stable hairpin structures have been applied to species with sequenced genomes, such as a number of nematodes, arthropods, and vertebrates (Table 1). Moreover, the development of novel techniques for directional cloning of small RNAs has led to the identification of many other miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

Nevertheless, the greatest progress came with the advent of high-throughput sequencing technologies and computational methods. Those technologies confirmed most of the miRNA predicted *in silico* in species with the genome reported, made it possible to find new and unexpected miRNAs, and contributed to the discovery *de novo* of miRNAs in species without the genome sequenced. Therefore, a consistent catalog of miRNAs is now available not only in drosophilids, but also in a selection of species, such as the malaria mosquito (*A. gambiae*), the yellow fever mosquito (*Aedes aegypti*), the pea aphid (*A. pisum*), the vector of West Nile virus (*Culex quinquefasciatus*), the jewel wasp (*Nasonia vitripennis*), the nigratory locust (*Locusta migratoria*), the honey bee (*Apis mellifera*), the flour beetle (*T. castaneum*), the silkworm (*Bombyx mori*), and the German cockroach (*B. germanica*) (http://www.mirbase.org; http://www.ncbi.nlm.nih.gov/geo) (Griffiths-Jones, 2006). Both approaches, based on computational methods and high-throughput sequencing, are discussed below.

2.4.1. Computational Methods

The most efficient computational methods for finding miRNA candidates were described in C. elegans (MiRscan) (Lim et al., 2003a) and D. melanogaster (miRseeker) (Lai et al., 2003). Both methods share conceptual similarities, such as structural and sequence similarity. MiRscan produces an initial set of candidates by sliding a 110-nucleotide window across the C. elegans genome and folding those segments that are filtered by the free energy and duplex length. Homologous hairpins are then identified by WU-BLAST in an additional genome which creates a reference set defining the standard features that will finally be used to score and rank all candidate hairpins. Nevertheless, MiRscan was not able to identify more than 50% of the previously known C. elegans miRNAs (Lim et al., 2003a). miRseeker was found to be more efficient at identifying genuine miRNAs in two fly species (D. melanogaster and Drosophila pseudoobscura) by taking into account the conservation across the hairpin (Lai et al., 2003). The method begins by identifying orthologous

Table 1 Algorithms Developed for a	miRNA Identification
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Program	Strategy	Species group	Authors/year
Grad et al.	RB	Nematodes	Grad <i>et al.</i> , 2003
MiRScan	RB	Nematodes, vertebrates	Lim <i>et al.</i> , 2003a, 2003b
miRseeker	RB	Insects (flies)	Lai <i>et al.</i> , 2003
Berezikov <i>et al.</i>	RB	Human	Berezikov et al., 2005
miPred	RB	Human	Jiang <i>et al.</i> , 2007
miRAlign	RB	Metazoan	Wang et al., 2005
ProMIR	HMM	Human	Nam et al., 2005
BayesMiRNAFind	NB	Nematodes, mammals	Yousef <i>et al.</i> , 2006
One-ClassMirnaFind	SVM, NB	Human, virus	Yousef et al., 2008
mirCoS-A	SVM	Mammals	Sheng <i>et al.</i> , 2007
mir-abela	SVM	Mammals	Sewer et al., 2005
triplet-SVM	SVM	Human	Xue <i>et al.</i> , 2005
RNAmicro	SVM	Metazoan	Hertel and Stadler, 2006
miPred	SVM	Human	Ng and Mishra, 2007
MiRFinder	SVM	Human, virus	Huang et al., 2007

HMM, hidden Markov model; NB; Naive Bayes; RB, rule based; SVM, support vector machine.

intergenic and intronic regions of those two fly genomes, and then folding those conserved sequences to identify and score the hairpin structures. The criteria for hairpin evaluation derive from a reference set of known miRNA genes of the two *Drosophila* species. The length of the hairpins and their minimum free energy were first evaluated, and then the distribution of divergent nucleotides was considered to score the candidates. The metrics consist in penalizing divergences depending on where they occur in the pre-miRNA hairpin, as the miRNA arm would tolerate less mutations than the miRNA* arm, which, by itself, would not tolerate more mutations than those observed in the loop region (Lai *et al.*, 2003).

The establishment of guidelines for the experimental validation and annotation of novel miRNA candidates became obviously necessary with the increasing quantity of miRNA genes being identified in various species (Ambros *et al.*, 2003). Thus, an initiative for organizing the information available on miRNA genes was then developed, leading to a database (miRBase, http://www.mirbase.org) where all data regarding miRNA sequences, targets, and gene nomenclature are deposited (Griffiths-Jones *et al.*, 2008).

The large amount of miRNA data available in databases led to the development of a second generation of algorithms based on machine-learning methods. The approach consists in a learning process that identifies the most relevant characteristics and rules from a positive set of miRNA hairpins. Various machine-learning algorithms have been used for miRNA discovery (**Table 1**), the most common being Naïve Bayes (Yousef *et al.*, 2006), support vector machines (Yousef *et al.*, 2008 and references therein), hidden Markov models (HMM) (Nam *et al.*, 2005), genetic programming (Brameier and Wiuf, 2007), and random walks (Jiang *et al.*, 2007).

All these methods contributed somehow to the identification of new miRNAs, despite considerable differences in their trade-off between specificity and sensitivity. The criteria used in all of them were based on actual knowledge of the miRNA biogenesis, and features identified from known miRNAs conserved in at least two species. Indeed, there must be a great number of non-conserved miRNA genes still to be discovered, which may have characteristics and expression profiles substantially different from those of canonical miRNAs. However, the development of a new generation of sequencing technologies is changing the way of thinking about scientific approaches in all fields of biological sciences (Metzker, 2010), including the strategies to find new miRNAs in any species, even those whose genome is not sequenced yet.

2.4.2. High-Throughput Sequencing

Deep-sequencing technologies have created a new paradigm in detecting low-expression or tissue-specific miRNAs, as well as non-canonical and species-specific ones. The most effective algorithms published so far are miRDeep (Yang *et al.*, 2010), MIReNA (Mathelier and Carbone, 2010), and deepBase (Friedlander *et al.*, 2008). Despite varying slightly in their workflow, their general strategy is similar, combining mapping and filtering sequences based on genome annotation, sequence and structure patterns, and properties of miRNA biogenesis.

The identification of miRNAs through deep-sequencing methods is rapidly increasing the catalogs of small RNA sequences for many species from a variety of taxonomic groups. Currently, all deep-sequencing datasets are deposited in the GEO (Gene Expression Omnibus) database at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/geo). At the date of writing (January 2011), there are at least 193 studies of high-throughput sequencing of small RNAs from different eukaryotic species in the GEO database, and the number of records for each.

Most of the 14 insect species included in **Table 2** have the genome sequenced, or at least have a closely related species with an available genome (e.g., *A. albopictus* and *C. quinquefasciatus*). Two species, *L. migratoria* and *B. germanica*, have no genome sequence available, and identification of miRNAs from deep-sequencing data becomes challenging because none of the methods mentioned above were designed to analyze deep-sequencing data without

 Table 2
 Insect Species and Number of Records Found in

 the GEO Database Related to Studies of miRNA Identification

Order	Species	Number of records
Diptera		
	Drosophila melanogaster	21
	Drosophila simulans	1
	Drosophila erecta	1
	Drosophila pseudoobscura	1
	Drosophila virilis	1
	Aedes albopictus	1
	Culex quinquefasciatus	1
Lepidoptera		
	Bombyx mori	2
Hymenoptera		
	Camponotus floridanus	1
	Harpegnathos saltator	1
	Apis mellifera	1
Hemiptera		
	Acyrthosiphon pisum	1
Orthoptera		
	Locusta migratoria	1
Dyctioptera		
	Blattella germanica	1

using a genome sequence as a reference, and the diversity of small RNA types is remarkably high. However, strategies that can identify previously described miRNAs, as well as novel miRNAs on the basis of the number of reads and hairpin features, have recently been proposed (Wei et al., 2009). Genome-independent approaches for miRNA discovery show that we still have a poor understanding of the small RNA world and its regulatory mechanisms in the cell. For example, in the locust L. migratoria (Wei et al., 2009) and in the cockroach B. germanica (Cristino et al., 2011), sequence read numbers corresponding to miRNA*s were higher than those corresponding to the mature miRNA. Another original finding has been reported in Drosophila species (Berezikov et al., 2010), where some miRNA precursors seem not to be processed by RNase III only, given that the usual one- to two-nucleotide 3' overhang does not occur in some sequences represented by a high number of reads.

2.4.3. miRNA Classification

As stated above, identification efforts have led to the description of an impressive number of miRNAs in animals, plants, green algae, fungi, and virus (Griffiths-Jones et al., 2008), and different attempts to classify such a high diversity into families based on structural coincidences have been carried out. As the pattern of nucleotide substitution in miRNA genes is apparently shaped by selective pressures, and considering that the seed is the most important region from a functional point of view (Brennecke et al., 2005; Bartel, 2009), miRNA classification is based on this region. Regarding metazoans, 858 miRNA families are deposited in the miRBase database (v16.0) (Griffiths-Jones et al., 2008), and 254 (30%) of these families are found in at least five species. These records will change with further high-throughput sequencing experiments, but present data indicate that most of the miRNA families (a total of 562) are found in vertebrates, followed by insects (178 families reported), and then by other metazoan that are phyllogenetically more basal, such as cnidaria, porifera, hemichordata, echinodermata, urochordata, cephalochordata, and nematoda (118 families in all).

The seed region can be more or less conserved in different miRNA families. A good example of a well-conserved seed region is observed in the miRNAs miR-100, miR-125, and let-7 (Behura, 2007). As stated above (see also Figure 3), these three miRNAs are often coded by the same polycistronic pri-miRNA that has a conserved organization from invertebrates to vertebrates, which suggest that it is an ancestral pri-miRNA. As expected, the seed region of these miRNAs is highly conserved (Figure 6). There are insect-specific miRNA families whose seed region is also very well conserved, as for instance bantam miR-2 and miR-3 (Figure 6). The conservation of the seed region occurs not only among paralogous sequences, resulting from intraspecific gene duplication, but also among orthologous sequences arising from speciation events. Of note, the conservation of the seed region is critical for the recognition of mRNA targets, thus the classification of miRNAs into families on the basis of the seed not only contains structural information, but may also reflect functional regularities.

2.5. Target Prediction

In animals, the functional duplexes miRNA:mRNA can occur in a variety of structures where short complementary sequences can be interrupted by gaps and mismatches (Brennecke *et al.*, 2005; Bartel, 2009). Thus, most computational methods have been developed to find target sequences based on the complementarity between the miRNA seed sequence and the mRNA sequence. Several computational approaches estimate the likelihood of miRNA:mRNA duplex formation, mainly based on



Figure 6 Conservation of miRNA genes on the region corresponding to mature miRNAs in metazoan and insects. The sequence logo is constructed based on the alignment of various miRNA sequences representing the level of nucleotide conservation in each position. The squares indicate the canonical seed regions located at nucleotides 2–8.

sequence complementarity, thermodynamic stability, and evolutionary conservation of the sequence among species (**Table 3**). Machine learning approaches are also used for miRNA target identification. These methods usually combine one or more of the traditional procedures (seed complementarity, thermodynamic stability, and cross-species conservation) with more elaborated probabilistic models (**Table 3**). Also, a new generation of algorithms is integrating high-throughput expression data and computational predictions (Huang *et al.*, 2007; Hammell *et al.*, 2008; van Dongen *et al.*, 2008; Wang and El Naqa, 2008; Bandyopadhyay and Mitra, 2009; H. Liu *et al.*, 2010; Sturm *et al.*, 2010).

To date, miRNA target prediction has been mainly performed by computational approaches, and large numbers of targets have been predicted for most species with the genome sequenced (Bartel, 2009). As a general figure, predictions have suggested that a single miRNA can target 200 mRNAs on average in vertebrates (Krek *et al.*, 2005), whereas in *D. melanogaster* a single miRNA may regulate 54 genes on average (Grun *et al.*, 2005).

2.5.1. microCosm, TargetScan, and PicTar

The miRBase database links miRNAs to targets using microCosm (http://www.ebi.ac.uk/enright-srv/microcosm/), TargetScan (Lewis *et al.*, 2005; Grimson *et al.*, 2007; Friedman *et al.*, 2009) and PicTar (Lewis *et al.*, 2005;

Grimson *et al.*, 2007; Friedman *et al.*, 2009) prediction systems. These are therefore the most currently used, and are detailed below.

microCosm, formerly known as miRBase Targets, predicts miRNA targets in the UTR regions of animal genomes from Ensembl database (Hubbard et al., 2007; Flicek et al., 2008). It uses the miRanda algorithm to calculate a score across the miRNA vs UTR alignment (Enright et al., 2003; John et al., 2004; Betel et al., 2008); the energy for the thermodynamic stability of a miRNA: mRNA duplex is calculated by the Vienna RNA folding routines (http://www.tbi.univie.ac.at/RNA/), and the P-values are computed for all targets following the statistical model implemented in RNAhybrid (Rehmsmeier et al., 2004). The Miranda algorithm (Enright et al., 2003; John et al., 2004) is basically divided into three steps. In the first step the miRNAs are aligned against the 3' UTR sequences of the targets, allowing for G: U pairs and short indels. The method does not rely on seed matches, but increases the scaling score for complementarity at the 5' end of the miRNA. The second step computes the thermodynamic stability of the miRNA:mRNA duplex, and the final step reduces the false-positive rate by considering only targets with multiple sites.

TargetScan was the first algorithm that used the concept of seed matches in target prediction (Lewis *et al.*, 2003, 2005). The method only uses miRNAs conserved across different species to scan corresponding 3' UTR sequences.

Algorithm	Strategy	Species group	Authors/year
TargetScan	RB	Vertebrates	Lewis <i>et al.</i> , 2003
TargetScanS	RB	Vertebrates	Lewis et al., 2005
miRanda	RB	Insects (flies), Human	Enright et al., 2003; John et al., 2004
Diana-microT	RB	Nematodes	Kiriakidou et al., 2004
RNAhybrid	RB	Insects (flies)	Rehmsmeier <i>et al.</i> , 2004
MovingTargets	RB	Insects (flies)	Burgler and MacDonald, 2005
MicroInspector	RB	Any species	Rusinov <i>et al.</i> , 2005
Nucleus	RB	Insects (flies)	Rajewsky and Socci, 2004
EIMMo	RB	Nematodes, Insects (flies), Vertebrates	Gaidatzis et al., 2007
TargetBoost	BT	Nematodes, Insects (flies)	Saetrom et al., 2005
PicTar	HMM	Nematodes, Insects (flies), Vertebrates	Krek <i>et al.</i> , 2005
RNA22	MC	Nematodes, Insects (flies), Vertebrates	Miranda et al., 2006
MicroTar	PD	Any species	Thadani and Tammi, 2006
PITA	PD	Nematodes, Insects (flies), Vertebrates	Kertesz <i>et al.</i> , 2007
NBmiRTar	NB	Metazoa	Yousef <i>et al.</i> , 2007
miTarget	SVM	Metazoa	Kim <i>et al.</i> , 2006
MiRTif	SVM	Metazoa	Yang <i>et al.</i> , 2008
mirWIP	E	Nematodes	Hammell et al., 2008
Sylamer	E	Metazoa	van Dongen <i>et al.</i> , 2008
GenMiR++	BL, E	Metazoa	Huang et al., 2007
SVMicrO	SVM, E	Mammals	H. Liu <i>et al.</i> , 2010
TargetMiner	SVM, E	Human	Bandyopadhyay and Mitra, 2009
MirTarget2	SVM, E	Metazoa	Wang and El Naqa, 2008
TargetSpy	BT, E	Insects (flies), Human	Sturm et al., 2010

Table 3 Algorithms Developed for Predicting miRNA Targets

BL, Bayesian learning; BT, Boosting technique; E, integration of expression data; HMM, hidden Markov model; MC, Markov chain; PD, pattern discovery; RB, rule based; SVM, support vector machine.

The algorithm defines the seed matches as short segments of seven nucleotides that must have a stringent complementarity to the two to eight nucleotides of the mature miRNA. Then, the remaining miRNA sequence is aligned to the target site, allowing for G: U pairs; the free energy to form a secondary structure in the duplex is predicted by a folding algorithm. A Z-score is calculated on the basis of the number of matches predicted in the same target sequence and respective free energies. Finally, the Z-score is used to rank the candidate targets for each species, and each species is processed in the same way.

PicTar uses a machine learning algorithm to rank target sequences using a HMM maximum likelihood score based on three main steps: (1) the seed matches must expand 7 nucleotides starting at position 1 or 2 in the 5' end of the miRNA; (2) the minimum free energy of miRNA: mRNA duplexes is used to filter the target sites; and (3) the target sites must locate in overlapping positions across the aligned corresponding 3' UTR sequences. The target sites that pass the three-step filter are then ranked by the HMM model, which calculates the score considering all segmentations of the target sequence into target sites and background, thus allowing the algorithm to account for multiple binding sites for a single miRNA, as well as several miRNAs targeting the same mRNA.

The current target predictions available in the miRBase by microCosm, TargetScan, and PicTar have some degree of overlap and also of discrepancy that can be due to alignment artifacts, different mRNA UTR and miRNA sequences, and intrinsic differences in the algorithms. In an attempt to provide more updated figures for the distribution of gene targets per miRNA and miRNA per gene target, we analyzed the data from target predictions available in the miRBase (Release 16; Sept 2010), comparing D. melanogaster with Homo sapiens and C. elegans. Results show that the three methods give different average numbers of miRNA-binding sites per mRNA target (19.6, 5.8, and 5.0 for MicroCosm, TargetScan, and Pic-Tar, respectively; Figure 7), as well as different numbers of mRNAs targeted by each miRNA (951, 395, and 426 for microCosm, TargetScan, and PicTar, respectively; Figure 8). The distribution of the number of miRNAbinding sites per mRNA target (Figure 7) is relatively similar among the three methods and the three species studied. Conversely, data on the number of mRNA targeted by an miRNA showed remarkable differences depending on the method, regarding not only the average values, but also and especially their pattern of distribution (Figure 8).

2.6. miRNA Functions

Insect model species can be studied through powerful genetic and genomic approaches, the paradigm being the fly *D. melanogaster*. Indeed, the first description of miRNA functions in insects was carried out in this species (Brennecke *et al.*, 2003), by looking at gain-of-function mutants (Lai, 2002; Lai *et al.*, 2005). miRNA functions



Figure 7 Frequency of the number of miRNA-binding sites in the 3^r UTR of target mRNAs in *Homo sapiens*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, calculated with the three prediction methods available in miRBase: microCosm, TargetScan, and PicTar (Release 16; September 2010).



Number of mRNAs predicted to be targeted by a miRNA

Figure 8 Frequency of the number of mRNAs predicted to be targeted a miRNA in *Homo sapiens*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, calculated with the three prediction methods available in miRBase: microCosm, TargetScan, and PicTar (Release 16; September 2010).

are currently being demonstrated by mutating the genes coding for the miRNAs under study, overexpressing the miRNA of interest, or silencing it using specific antimiRNAs, and then studying the resulting phenotype. Predicted targets may also be validated by the above methods, including the quantification of the expression of the given target, as well as using *in vitro* systems with luciferase reporter target constructs, where binding of the miRNA to the target sequence is detected by luciferase activity and quantified with colorimetry.

In most cases, functions may be suggested by highthroughput sequencing comparisons in different developing stages, in different organs of the same stage, or in different physiological situations. Studies of this type have been carried out in the silkworm B. mori (differences in tissue expression and in different developing stages) (Cao et al., 2008; S. Liu et al., 2010), the pea aphid A. pisum (differences in different morphs) (Legeai et al., 2010), the honey bee A. mellifera (differences between queens and workers) (Weaver et al., 2007), the migratory locust L. migratoria (differences between migratory and solitary phases) (Wei et al., 2009), and the German cockroach B. germanica (differences between metamorphic and nonmetamorphic instars) (Cristino et al., 2011). Microarray analysis or detailed studies on the developmental expression profiles of particular miRNAs can also suggest their respective functions (Aravin and Tuschl, 2005; Weaver et al., 2007; He et al., 2008; Yu et al., 2008).

Silencing Dicer-1 expression by RNAi is also a useful approach to studying the influence of the whole set of miRNAs in a given process. This has been achieved in *D. melanogaster*, either *in vivo*, showing, for example, that Dicer-1 plays a general role in ovarian development (Jin and Xie, 2007), or in *Drosophila* cultured cells, where the depletion of Dicer-1 affected the development in both somatic and germ lineages (Lee *et al.*, 2004b). More recently, Dicer-1 depletion by RNAi has been used in the German cockroach, *B. germanica*, to demonstrate the key role of miRNAs in hemimetabolan metamorphosis (see below).

Regarding the functions of particular miRNAs, the data available indicate that most of them appear to be involved in the fine-tuning of biological processes by modulating a precise dosage of regulatory proteins. Probably, they provide robustness to the whole program of gene expression (Hornstein and Shomron, 2006) and resilience to environmental fluctuations, as in the case of miR-7 studied by Li and colleagues (X. Li *et al.*, 2009). However, as revealed by recent general reviews (Bushati and Cohen, 2007; Jaubert *et al.*, 2007), information is still fragmentary, heavily concentrated in the *D. melanogaster* model, and focused on a few biological processes, as detailed in the text below and in **Table 4**, which summarizes cases

Table 4 Functions of miRNA Demonstrated Experimenta

Function/process	miRNA	Target involved	Authors/year
Cell division of the germinal stem cells	bantam		Hatfield <i>et al.</i> , 2005; Shcher- bata <i>et al.</i> , 2007
Cell division of the germinal stem cells	miR-7, miR-278, miR309	Dacapo	Yu <i>et al.</i> , 2009
Germ-line differentiation	miR-7	bam	Pek <i>et al.</i> , 2009
Stem cells differentiation	miR-184	Saxophone	lovino <i>et al.</i> , 2009
Axis formation in the egg chamber	miR-184	Gurken	lovino <i>et al.</i> , 2009
Formation of the head and posterior abdominal segments in the embryo	miRs-2/13		Boutla <i>et al.</i> , 2003
Embryo segmentation	miR-31, miR-9		Leaman <i>et al.</i> , 2005
Embryo growth	miR-6		Leaman <i>et al.</i> , 2005
Formation of embryonic cuticle	miR-9		Leaman <i>et al.</i> , 2005
Photoreceptor differentiation	miR-7	Yan	Li and Carthew, 2005
Formation of sensory organs	miR-9a	Senseless	Li <i>et al.</i> , 2006
Location of CO ₂ neurons	miR-279	Nerfin-1	Cayirlioglu <i>et al.</i> , 2008
Protection of sense organs from apoptosis	miR-263a/b	Hid	Hilgers <i>et al.</i> , 2010
Muscle differentiation	miR-1	Delta	Kwon <i>et al.</i> , 2005
Muscle differentiation	miR-133	nPTB	Boutz <i>et al.</i> , 2007
Growth	bantam		Hipfner <i>et al.</i> , 2002; Edgar, 2006; Thompson and Cohen, 2006
Tissue growth via insulin receptor signaling	miR-278		Teleman <i>et al.</i> , 2006
Growth via insulin receptor signaling	miR-8	U-shaped	Hyun <i>et al.</i> , 2009
Modulation of ecdysteroid pulses	miR-14	EcR	Varghese and Cohen, 2007
Neuromusculature remodeling during metamor- phosis	let-7 (and miR-100, miR-125)		Sokol et al., 2008
Maturation of neuromuscular junctions during metamorphosis	let-7 (and miR-125)	abrupt	Caygill and Johnston, 2008
Wing formation	miR-9a	dLOM	Biryukova <i>et al.</i> , 2009
Wing formation	iab-4	Ultrabithorax	Ronshaugen et al., 2005
Regulation of circadian rhythms	bantam	clock	Kadener et al., 2009a
Regulation of brain atrophin	miR-8	atrophin	Karres <i>et al.</i> , 2007
Anti-apoptotic	Bantam, miR-2	hid	Brennecke <i>et al.</i> , 2003, 2005; Stark <i>et al.</i> , 2005
Anti-apoptotic in D. melanogaster	miR-14	Drice	Xu <i>et al.</i> , 2003
Anti-apoptotic in Lepidopteran Sf9 cells	miR-14		Kumarswamy and Chandna, 2010
Anti-apoptotic in the embryo	miR-2, miR-13, miR-11	hid, grim, reaper, sickle	Leaman <i>et al.</i> , 2005

*All results refer to Drosophila melanogaster, except in the anti-apoptotic action of miR-14, which has been demonstrated also in Sf9 cells of the Lepidopteran Spodoptera frugiperda.

where the miRNA function has been demonstrated experimentally.

2.6.1. Germ-Line and Stem Cell Differentiation, Oogenesis

In *D. melanogaster*, cell division of the germinal stem cells (GSC) is under the control of different miRNAs. One of them, bantam, regulates the expression of specific mRNAs in the ovary, being involved in the maintenance of germinal stem cells (Hatfield *et al.*, 2005; Shcherbata *et al.*, 2007). Other miRNAs, like miR-7, miR-278 and miR-309, directly repress Dacapo mRNA through its 3' UTR, as demonstrated by Yu and colleagues (2009) using luciferase assays. These authors also suggest that bantam and miR-8 regulate Dacapo indirectly, controlling GSC

division; moreover, GSC deficient for miR-278 show a mild, but significant, reduction of cell proliferation. Depletion of miR-7 levels in GSC results in a perturbation of the frequency of Cyclin E-positive GSC, although the kinetics of cell division in miR-7 mutant GSC does not become reduced (Yu *et al.*, 2009).

Another miRNA that plays important roles in stem cell differentiation is miR-184. Depletion of miR-184 in *D. melanogaster* determines that females lay abnormal eggs and become infertile. Stem cell differentiation is impaired due to the increase of Saxophone protein levels. Later, during oogenesis, the absence of mir-184 impairs the axis formation of the egg chamber as a result of altering the expression of Gurken mRNA. In addition, the absence of miR-184 also affects the expression of pair-rule genes required for normal anteroposterior patterning and cellularization of the embryo (Iovino et al., 2009).

Finally, and also in *D. melanogaster*, miR-7 is involved in germ-line differentiation via *maelstrom* and *Bag-of-marbles* (*Bam*) gene products. Maelstrom regulates *Bam* via repression of miR-7, by binding to the miR-7 promoter region (Pek *et al.*, 2009); therefore, *D. melanogaster* mutants for *maelstrom* overexpress *Bam*, which leads to a deficient germ-line differentiation. As expected, a reduction in miR-7 expression rescues this phenotype (Pek *et al.*, 2009)

2.6.2. Embryo Patterning and Morphogenesis

After injecting anti-miDNA-2a and anti-miDNA-13a, *D. melanogaster* embryos exhibited defects in the head and posterior abdominal segments, including cuticle holes and denticle belt malformations. In view of the similarity of the induced phenotypes, Boutla and colleagues (2003) concluded that these related miRNAs, miR-2a and miR-13a, act on the same target genes, together with the also related miR-2b and miR-13b, which form a functional subgroup called miRs-2/13.

Leaman and colleagues (2005) injected antisense 2'O-methyl oligoribonucleotides targeting specific miR-NAs into early embryos of *D. melanogaster* in order to screen the function of these miRNAs. Results showed that embryos depleted for miR-31 and miR-9 completed development, but were affected by severe segmentation defects (**Figure 9**). Those injected with miR-9 antisense rarely formed any trace of cuticle, and did not show internal differentiation. Embryos depleted for miR-6 were generally smaller in size than controls and had fewer and abnormally large segments, thus suggesting that apoptotic processes had been enhanced.

2.6.3. Sensory Organs and Functions

In *D. melanogaster*, miR-7 has been localized in early photoreceptors during embryonic eye development. At this developmental stage, miR-7 stimulates photoreceptor differentiation through a reciprocal regulation with *yan*, a gene encoding a transcription factor involved in the differentiation of retinal progenitor cells (Li and Carthew, 2005).

Another miRNA involved in sensory organ development is miR-9. Through both loss-of-function and gainof-function analyses *in vivo*, Li and colleagues (2006) have reported that miR-9a is responsible for generating precise numbers of sensory organs in *D. melanogaster* embryos and adults. To accomplish this regulatory function, miR-9a represses the translation of *Senseless* mRNA through its 3' UTR region, thus ensuring a precise differential expression of this gene in sensory organ precursors and in the adjacent epithelial cells (Li *et al.*, 2006).

Neurons that sense CO_2 also provide an interesting case for study. They may have different locations depending on the species. In *D. melanogaster* they are located in the antenna, whereas in mosquitoes they are found in the maxillary palps. Cayirlioglu and colleagues (2008) observed that loss of miR-279 in *D. melanogaster* determines that CO_2 neurons change their location from the antennae to the maxillary palps. The authors suggest that miR-279 downregulates Nerfin-1, a specific target for this miRNA, thus preventing the development of CO_2 neurons in the maxillary palps (Cayirlioglu *et al.*, 2008).

An example of miRNA that ensures developmental robustness during apoptotic tissue pruning is miR-263a/b (Hilgers *et al.*, 2010), which protects sense organs during apoptosis by directly acting upon, and limiting the expression of, the pro-apoptotic gene *hid.* This property



Figure 9 Effects of depletion of miR-31 in *Drosophila melanogaster* embryos. (A) and (E) show Darkfield images of cuticle preparations; (B), (C), (F), and (G) are confocal images of Eve (red) and Ftz (green) stainings; and (D) and (H) *hairy* RNA *in situ* hybridization of blastoderm (2.5-h) embryos. (A)–(D) correspond to controls, and (E)–(H) to *miR-31* antisense-injected embryos. The latter show cuticle defects ranging from partial fusion to complete loss of segments (E). In controls (B) and (C), Eve and Ftz are expressed in seven largely non-overlapping stripes, while *miR-31* antisense-injected embryos (F) and (G) show fewer and weaker stripes that often bleed into each other. The *hairy* transcript pattern also shows fewer stripes (H), indicating that pattern formation is affected upstream of the primary pair rule genes. From Learnan and colleagues (2005), reprinted with permission from *Cell* (Elsevier).

of some miRNAs to buffer fluctuating levels of gene activity makes them well suited to serve a protective function during development (Hilgers *et al.*, 2010).

2.6.4. Muscle Differentiation

In *D. melanogaster*, miR-1, which is one of the best conserved miRNAs in animals, is specifically expressed in the mesoderm during early embryogenesis, and in myogenic precursors and muscle cells in late embryos (Sokol and Ambros, 2005). Depletion of miR-1 using genetic approaches, or by treatment with 2'O-methyl antisense oligonucleotides, resulted in lethality, which implies that miR-1 has essential functions in mesodermally derived tissues (Nguyen and Frasch, 2006).

By analyzing *D. melanogaster* mutants devoid of miR-1, Kwon *et al.* (2005) assessed the essential role of miR-1 for muscle differentiation. They showed that miR-1 regulates the determination of specific cardiac and somatic muscle lineages from pluripotent progenitor cells in early embryogenesis. The Delta protein, a ligand for the Notch signaling pathway, was identified as an miR-1 target in cardiac progenitor cells (Kwon *et al.*, 2005).

Another well-conserved miRNA is miR-133, which is expressed in muscle cells together with miR-1. In *D. melanogaster* embryos, miR-133 plays a key role in controlling alternative splicing during muscle formation, and defining the properties of differentiated muscle cells, through repressing the expression of the splicing factor nPTB during myoblast differentiation into myotubes (Boutz *et al.*, 2007). The results of Boutz and colleagues not only indicate miR-133 directly downregulates a key factor during muscle development, but also establish a role for microRNAs in the control of a developmentally dynamic splicing program.

2.6.5. Growth

In *D. melanogaster*, loss-of-function mutations of the bantam locus are lethal at the early pupal stage, whereas hypomorphic combinations of bantam mutant alleles give rise to adult flies that are smaller than controls (**Figure 10**) and that have deficiencies in fertility (Hipfner *et al.*, 2002). Conversely, overexpression of bantam induces tissue overgrowth due to an increase in cell number. Bantam expression appears to be regulated by the gene *Yorkie*, thus controlling organ growth during development (Edgar, 2006; Thompson and Cohen, 2006).

Related to growth, and also in *D. melanogaster*, miR-278 has been implicated in insulin receptor (InR) signaling, thus contributing to regulation of the energy balance mainly by controling insulin responsiveness. Overexpression of miR-278 promotes tissue growth in the eye and wing imaginal disks, whereas its deficiency leads to a reduction of fat body mass, which is reminiscent of the effect of impaired InR signaling in adipose tissue; the action of miR-278 could be produced through the regulation of *expanded* gene transcripts (Teleman *et al.*, 2006).

More recently, Hyun and colleagues (2009) have reported that miR-8 and its target, U-shaped (USH), regulate body size in *D. melanogaster*. miR-8 null flies are smaller in size and defective in insulin signaling in the fat body. USH inhibits PI3K activity, thus suppressing cell growth. Fat-body-specific expression and clonal analyses showed that miR-8 activates PI3K, thereby promoting fat-cell growth cell-autonomously, and enhancing organismal growth non-cell-autonomously (Hyun *et al.*, 2009).

2.6.6. Metamorphosis: Ecdysteroids and Juvenile Hormone

In insects, molting and metamorphosis are controlled by juvenile hormones and ecdysteroids, usually 20-hydroxyecdysone. Simultaneous expression of miR-125 and let-7 during *D. melanogaster* post-embryonic development is synchronized with the high titer of ecdysteroid pulses that initiate metamorphosis (Bashirullah *et al.*, 2003; Sempere *et al.*, 2003), which suggests that ecdysteroids might regulate the expression of these two miRNAs.

Bashirullah and colleagues (2003), however, showed that miR-125 and let-7 expression is neither dependent on the Ecdysone receptor (EcR) nor inducible by 20-hydroxyecdysone in larval organs incubated *in vitro*. The same authors reported that the expression of both miRNAs can be induced by 20-hydroxyecdysone in *Drosophila* Kc cells, although the induction is considerably delayed with respect to what is observed *in vivo* (Bashirullah *et al.*, 2003). The conclusion of these experiments is that the action of 20-hydroxyecdysone in Kc cells might be indirect, and that miR-125 and let-7 should be directly induced by an unknown temporal signal distinct from the well-known ecdysteroid-EcR cascade.

In a parallel paper, Sempere and colleagues (2003) followed a different approach to study the influence of ecdysteroids on the expression of miR-125, let-7, and miR-100, which are upregulated after the ecdysteroid pulse, as well as of miR-34, which is downregulated. They used the temperature-sensitive ecd¹ strain that is impaired in ecdysteroid synthesis, and they showed that in ecd¹ specimens blocked from pupariation by a transfer at 29°C, miR-125, let-7, and miR-100 were detected at much lower levels, whereas miR-34 was detected at much higher levels, compared with the wild type. Sempere and colleagues (2003) also studied the possible role of Broad complex, an early inducible gene in the ecdysteroid cascade, using npr⁶ specimens, which lack all Broad complex factors. Results showed that miR-125, let-7, and miR-100 were detected at much lower levels (and miR-34 at much higher levels) in homozygous npr⁶ specimens than in $npr^{6}/+$ or wild type specimens. With the same experimental approach, Sempere and colleagues (2003)



Figure 10 In *Drosophila melanogaster*, loss-of-function mutations of the bantam locus are lethal at the early pupal stage, whereas hypomorphic combinations of bantam mutant alleles give rise to adult flies that are smaller than controls. Panel (A) shows the reduced body size of male and female flies of $ban^{L1170}/ban^{\Delta 1}$ (defective for bantam), with respect to $ban^{\Delta 1}/TM2$ siblings (control). Panel (B) compares wing sizes in both combinations. In the overlay, the $ban^{L1170}/ban^{\Delta 1}$ wing is shown in red and the $ban^{\Delta 1}/TM2$ wing in green. From Hipfner and colleagues (2002), reprinted with permission from the Genetics Society of America.

concluded that ecdysteroids and Broad complex activity are required for temporal upregulation of miR-125, let-7, and miR-100, and downregulation of miR-34. Additional experiments carried out by these authors with *Drosophila* S2 cells showed that incubation times longer than 30 h with 20-hydroxyecdysone correlated with increased levels of miR-125, let-7, and miR-100, whereas miR-34 was detected at very low levels at all times studied. Moreover, the addition of methoprene, a juvenile hormone analog, enhanced the expression of miR-34 and reduced the ecdysteroid-stimulatory effect on the expression of miR-125, let-7, and miR-100 (Sempere *et al.*, 2003). In these experiments, Broad complex was shown to be necessary for enhancing the activity of 20-hydroxyecdysone.

Discrepancies between the two papers (Bashirullah et al., 2003; Sempere et al., 2003) look more apparent

than real, given that the respective sets of results, which emerge from quite different experimental approaches, are not incompatible. Indeed, those of Sempere and colleagues (2003) do not discard an indirect action of ecdysteroids, which is the hypothesis postulated by Bashirullah and colleagues (2003).

A miRNA clearly associated to ecdysteroid pulses in *D. melanogaster* is miR-14. In this fly, ecdysteroid signaling through the EcR seems to act via a positive autoregulatory loop that increases EcR levels, thus optimizing the effect of ecdysteroid pulses. In this context, miR-14 modulates this loop by limiting the expression of EcR, whose mRNA contains three miR-14 sites in the 3' UTR. In turn, ecdysteroid signaling, through EcR, downregulates miR-14. This modulatory action of miR-14 may be crucial due to the intrinsic lability of the positive

autorregulatory loop that controls ecdysteroid signaling (Varghese and Cohen, 2007).

2.6.7. Metamorphosis: Morphogenesis

Work by Sokol and colleagues (2008) showed that the *D. melanogaster* let-7-Complex locus (let-7-C, comprising let-7, miR-100, and miR-125; see **Figure 3**) is mainly expressed in the pupal and adult neuromusculature. let-7-C knockout flies look morphologically normal, but display defects in different adult behaviors (like flight and motility) and in fertility. Importantly, their neuromusculature clearly shows juvenile features, which suggests that an important function of let-7-C is to ensure the appropriate remodeling of the abdominal neuromusculature during the larval-to-adult transition. The study also showed that this function is carried out predominantly by let-7 alone (Sokol *et al.*, 2008).

In a related work, Caygill and Johnston (2008) obtained a *D. melanogaster* mutant that lacks let-7 and miR-125 activities and shows a pleiotropic phenotype that arises during metamorphosis. These authors showed that the loss of let-7 and miR-125 results in temporal delays in the terminal cell-cycle exit in the wing, and in the maturation of neuromuscular junctions of imaginal abdominal muscles. The authors focused on the latter process by identifying the *abrupt* (*ab*) gene (which encodes a nuclear protein) as a let-7 target, and by providing evidence showing that let-7 regulates the maturation rate of abdominal neuromuscular junctions during metamorphosis by regulating *ab* expression (Caygill and Johnston, 2008).

Wing morphogenesis has been studied by Biryukova and colleagues (2009), who described that miR-9a regulates D. melanogaster wing development through a functional target site in the 3' UTR of the LIM only (dLOM) mRNA. dLMO is a transcription cofactor that directly inhibits the activity of Apterous, the factor required for the proper wing dorsal identity. Deletions of the 3' UTR that remove the miR-9a site generate gain-of-function dLMO mutants associated with high levels of dLMO mRNA and protein. These mutants lack wing margins, a phenotype that is characteristic of null miR-9a mutants. Of note, miR-9a and dLMO are co-expressed in wing disks and interact genetically for controlling wing development; thus, the absence of miR-9a results in overexpression of dLMO, while gain-of-function miR-9a mutant suppresses dLMO expression. The data suggest that miR-9a ensures a precise dosage of dLMO during D. melanogaster wing development (Biryukova et al., 2009).

Another miRNA involved in wing morphogenesis of *D. melanogaster* is iab-4. Sequence analysis suggested that iab-4 could regulate *Ultrabithorax* (*Ubx*), and expression pattern studies of iab-4 and *Ubx* showed that they are complementary in critical developmental moments. Direct evidence for an interaction between iab-4 and *Ubx*

was obtained with luciferase assays. Finally, ectopic expression of iab-4 miRNA in haltere disks caused a homeotic transformation of halteres to wings, which occurs when *Ubx* expression is reduced (Ronshaugen *et al.*, 2005).

As stated above, RNAi experiments that reduced Dicer-1 expression in the last instar nymph of *B. germanica* depleted miRNA levels, and the next molt, instead of giving the adult stage, gave supernumerary nymphs. These were morphologically similar to the supernumerary nymphs obtained after treating the last instar nymph with juvenile hormone (**Figure 11**). The RNAi experiments with Dicer-1 indicate that miRNAs are crucial for hemimetabolan metamorphosis (Gomez-Orte and Belles, 2009).

2.6.8. Behavior

A recent paper by Kadener and colleagues (2009a) addresses the contribution of miRNAs to the regulation of circadian rhythms. The authors first knocked down the miRNA biogenesis pathway in D. melanogaster circadian tissues, which severely affected behavioral rhythms, thus indicating that miRNAs function in circadian timekeeping. To identify miRNA-mRNA pairs that might be important for this regulation, immunoprecipitation of Ago-1, followed by microarray analysis, led to identification of a number of mRNAs presumably under miRNA control. These included three core clock mRNAs: clock; vrille; and clockworkorange. To identify miRNAs involved in circadian timekeeping, the authors inhibited miRNA biogenesis in circadian tissues and then carried out a tiling array analysis. Behavioral and molecular experiments showed that bantam has a role in the core circadian pacemaker, and S2 cell biochemical assays indicated that bantam regulates the translation of clock by targeting three sites in the clock 3' UTR (Kadener et al., 2009a).

In a work addressed to study of the role of miR-8 in *D. melanogaster*, Karres and colleagues (2007) identified atrophin (also known as grunge) as a direct target of miR-8. miR-8 mutant phenotypes show high levels of apoptosis in the brain, and behavioral defects, like impaired capability for climbing, which are attributable to elevated atrophin activity. Decrease of atrophin levels in miR-8 regulation is detrimental, which points to a sort of "tuning target" relationship between them (Karres *et al.*, 2007).

2.6.9. Polyphenism, Caste Differentiation, and Sexual Differences

Legeai and colleagues (2010) suggested that miRNA might participate in the regulation of aphid polyphenism, and studied the expression of miRNA in different female morphs of *A. pisum* using microarray approaches. Most (95%) of the miRNA tested (n = 149) had similar expression in different morphs, but some of them, including



Figure 11 Inhibition of *Blattella germanica* metamorphosis after impairing miRNA maturation by depleting Dicer-1 expression with RNAi approaches in sixth (last) instar nymph. Dorsal and ventral view of normal sixth instar nymph (A, B), normal adult (C, D) and seventh instar supernumerary nymphoid (E, F) resulting from metamorphosis inhibition. The nymphoids resemble those obtained after treating the last instar nymph with juvenile hormone (G). Photos from Albert Masó; data from Gomez-Orte and Belles (2009).

miR*s, were differentially expressed – like let-7 and miR-100, which were upregulated in oviparae specimens, and miR2a-1, which was downregulated. The comparison between two parthenogenetic morphs gave three miR-NAs (miR-34, miR-X47, and miR-X103) and two miR*s (miR307* and miRX52*) that showed differential expression. While miR307* was upregulated in virginoparae, the others were downregulated with respect to the sexuparae morph.

Using total reads from Solexa deep sequencing, Wei and colleagues (2009) compared miRNA expression in the gregarious and solitary phases of the migratory locust, L. migratoria. In the gregarious phase, canonical miRNAs were expressed at levels between 1.5- and 2-fold higher than in the solitary phase; the most prominent differences were found in miR-276, miR-125, miR-1, let-7, and miR-315. Interestingly, miR-1 is a muscle-specific miRNA and miR-315 is a potent Wingless signaling activator, at least in D. melanogaster; therefore, the differences in flying power between gregarious and solitary locusts may be related by the action of these two miRNAs. However, most of the differences concern new unannotated small RNAs, which are much more abundant in the solitary phase, although the functions of these miRNA candidates remain unknown.

In the honey bee *A. mellifera*, expression profiles of miRNAs in workers and queens have been compared using quantitative RT-PCR, in adult body parts (head, abdomen, thorax) as well as in the whole body of the pupal stage (Weaver *et al.*, 2007). Results highlighted the differential expression, between queens and workers, in the abdomen,

which is probably related to the location of the ovaries and the differential fecundity of the two castes. Regarding particular miRNAs, miR-71 shows strong expression in worker pupae; comparing adult body parts, miR-71 has a higher expression in the head and thorax of adult workers, whereas expression in the abdomen is higher in the queen caste. Conversely, miR-9a is highly expressed in the thorax and abdomen of workers, while their expression levels are the highest in the thorax of workers.

In the silkworm B. mori, Liu and colleagues studied the expression of miRNA adult males and females using microarray approaches, and found that the expression of some 20 miRNAs was significantly higher in the body wall of males (S. Liu et al., 2010). This differential expression was assessed for a selection of 10 miRNAs (including bantam, miR-1, miR-13a, and miR-2a) using Northern blot. Microarray analysis also revealed that the expression of 13 miRNAs was significantly higher in ovaries, whereas only 4 were differentially expressed in testes. Differences between sexes were also found in other tissues, including Malpighian tubules, head, midgut, fat body, or silk gland. However, the authors pointed out that differences in miRNA expression might be due to individual differences in the metabolic state, because the expression of some of these miRNAs is influenced by nutritional status (Cheung et al., 2009).

2.6.10. Response to Biological Stress

Larvae of the moth *Lymantria dispar* show differentiated miRNA expression after wasp parasitization (Gundersen-Rindal and Pedroni, 2010). Microarray studies revealed that miR-1, miR-184, and miR-277 are highly upregulated in larval hemocytes, whereas miR-279 and let-7 are highly downregulated. Expression changes were assessed in hemolymph, fat body, brain, and midgut from infected larva, with respect to controls, using qRT-PCR. Of all the tissues analyzed from parasitized specimens, the midgut was the one that showed least miRNA activity. miR-1 was upregulated in all tissues from parasitized specimens, whereas miR-277 was the most strongly upregulated in the fat body. Expression of miR-279 was variable in the different tissues; it was remarkably upregulated in fat body but clearly downregulated in hemolymph, and it had a negligible expression in brain and midgut. Two human herpes virus-associated miRNAs (hcmv-miR-UL70 3p and kshv-miR-K12-3) were upregulated in hemocytes of parasitized L. dispar (Gundersen-Rindal and Pedroni, 2010). These are among many miRNAs that have been hypothesized to act as suppressors of the immediate and early genes that respond to a viral infection (Murphy et al., 2008).

The response of miRNAs to an infection has also been studied in mosquitoes. In A. gambiae, expression of miR-34, miR-1174, and miR-1175 decreases after Plasmodium infection, while that of miR-989 increases (Winter et al., 2007). Minor changes in miRNA expression have been observed in C. quinquefasciatus after West Nile virus infection (Skalsky et al., 2010), although miR-989 showed a 2.8-fold downregulation and miR-92 appeared somewhat upregulated. The expression of these two miR-NAs has been studied in different mosquito species, and results have shown that miR-989 expression is restricted to females, and predominantly to the ovary (Winter et al., 2007; Mead and Tu, 2008), although it was later detected in the midgut of Ae. aegypti (S. Li et al., 2009). miR-92 has been related to embryonic development in Ae. aegypti (S. Li et al., 2009) and B. mori (Liu et al., 2009). Results of deregulation of miR-989 and miR-92 suggested to Skalsky and colleagues (2010) that their targets participate in mediating flavivirus infection of the mosquito host.

2.6.11. Apoptosis

A group of *D. melanogaster* miRNAs including, miR-278, miR-14, bantam, and miR-2, regulate cell proliferation and apoptosis, targeting a number of pro-apoptotic genes, like *hid*, which is repressed by bantam and miR-2 miR-NAs (Brennecke *et al.*, 2003, 2005; Stark *et al.*, 2005).

One of the functions of mir-14 in *D. melanogaster* is suppressing cell death; therefore, loss of miR-14 is associated with a reduced lifespan, stress sensitivity, and increased levels of the apoptotic effector caspase Drice (Xu *et al.*, 2003). The same anti-apoptotic function has been found in Lepidopteran (*Spodoptera frugiperda*) Sf9 cells, where miR-14 is required for constitutive cell survival (Kumarswamy and Chandna, 2010). However, the results do not exclude that additional miRNAs might also contribute to regulating Lepidopteran cell survival and death.

Finally, experiments depleting miRNA functions by injection of miRNA antisense nucleic acids in early embryos, which permits systematic loss-of-function analysis *in vivo*, have identified the miR-2/13 family and miR-6 as controlling apoptosis during *D. melanogaster* embryonic development through post-transcriptional repression of the proapoptotic proteins hid, grim, reaper, and sickle (Leaman *et al.*, 2005).

2.7. Conclusions and Perspectives

There are reasons to believe that the still-scarce data available on miRNAs are just the tip of an iceberg. Nevertheless, rapidly expanding information is making it increasingly obvious that miRNAs' contribution to the genomic output is not a sort of genetic oddity or "transcriptional background noise," but a class of key post-transcriptional regulators of gene expression. Indeed, genomic regulation cannot be completely understood without incorporating the role of miRNAs, which constitute a regulatory layer that works in concert with the mRNA and protein network. However, the field of miRNA study is still in the development phase, and there are many aspects – the bulk of the iceberg – that require further research.

The miRNA machinery appears to be more complex than previously thought, and rapid progress is unveiling many unexpected details. An example concerning the mechanisms responsible for stabilized or reduced miRNA expression is the discovery of specific cis-acting modifications and trans-acting proteins that affect miRNA halflife, which are revealing new elements that contribute to their homeostasis (Kai and Pasquinelli, 2010), and the identification of Dicer-independent miRNA biogenesis pathways, such as those using the catalytic activity of Ago-2 (Cheloufi *et al.*, 2010; Cifuentes *et al.*, 2010). Therefore, recent data suggest that such mechanistic aspects will have more surprises revealed as we continue to expand our understanding of them.

Moreover, further studies are required to elucidate how miRNA genes are regulated. There are contributions studying the influence of transcription factors acting on the promoter region of miRNA genes, like that of Pek and colleagues (2009) on the aforementioned repressor action of Maelstrom on the miR-7 promoter region. However, more studies in this line are needed if we wish to better understand the regulatory mechanisms mediated by miRNAs. Faster progress seems predictable in the field of cataloging miRNAs. The challenge is to find the unexpected, non-conserved miRNAs, and the new generation of algorithms will have to combine not only high-throughput approaches and powerful computational methods, but also expression data, genomic location, and structural and sequence features, as approached in the studies of Brennecke and Cohen (2003), Friedlander and colleagues (2008), and Mathelier and Carbone (2010).

miRNA target prediction started more than two decades ago with the serendipitous findings that emerged from miRNA target recognition (Wightman *et al.*, 1991, 1993; Lee *et al.*, 1993). The key principles were then applied to computational methods for miRNA target prediction (Bartel, 2009), and these methods soon allowed the prediction of hundreds of miRNA targets. However, computational prediction of miRNA targets still relies on the few principles defined more than 20 years ago, and, arguably, this will not help to unveil novel aspects of miRNA target mechanisms. Thus, unbiased approaches to studying the interaction of miRNA and target would be valuable in order to identify new principles of miRNA-target recognition, and to improve the systems for target prediction, as in the creative approach of Orom and Lund (2007).

Finally, the phase of predicting putative targets in silico following computational methods must be followed by experimental work to validate the predictions and to identify targets in vivo. In this line, specific miRNA silencing will be one of the most useful approaches, and entomologists and other non-biomedical researchers will benefit from the miRNA antagonists that are being designed in the context of biomedical studies in search of therapeutic agents against human diseases (Gao and Huang, 2009). In this sense, D. melanogaster and other insect species will continue to be the favorite models, given the advantages they offer, especially straightforward manipulation. Validation of targets will contribute to elucidating the place and role of miRNAs in the molecular network that regulates the development and homeostasis of biological processes. The networks describing the interaction of miRNAs, mRNAs, and proteins are presumably highly organized and complex, and their their study therefore represents a formidable challenge. However, it will be a worthwhile effort, because these networks are possibly the best approximation to the living world that is available with present means.

There is a fairly widespread opinion that proteins are what really matter in the functional landscape that shapes fitness, so transcript abundance is only useful as a mere proxy for the activity of the corresponding proteins (Feder and Walser, 2005). However, the expanding universe of small silencing RNAs (Ghildiyal and Zamore, 2009) and their widespread functional roles in genomic regulation show this to be yet one more old paradigm that is about to fall.

Acknowledgments

Financial support from the Ministry of Science and Innovation, Spain (Projects BFU2008-00484 to MDP and CGL2008-03517/BOS to XB) is gratefully acknowledged.

EDT has a post-doctoral contract from the CSIC (JAEdoc program) and MR a pre-doctoral research grant from the CSIC (JAE-pre program).Thanks are due to Stephen Cohen for providing **Figure 10**, and to Albert Masó for the photographs in **Figure 11**.

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