Int. J. Dev. Biol. 61: 165-170 (2017)
doi: 10.1387/ijdb.160271ht

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

microRNAs in *Drosophila* regulate cell fate by repressing single mRNA targets

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ABSTRACT Regulation of gene expression governs all aspects of the lifespan of the organism, such as embryonic development, stem cell differentiation, reproduction and aging. Among the most important regulators of these extremely complex processes are microRNAs (miRNAs), small non-coding RNAs that repress gene expression by binding to primary sequences on the mRNA of their target. Theoretically, the mere existence of a miRNA recognition sequence on a given mRNA is sufficient to generate a functional response. Since these short sequences are abundant, one miRNA can potentially bind to multiple targets, thus generating endless possible biological outcomes. However, is this really the case? Bioinformatics and molecular biology tools provide theoretical interaction predictions, but the data obtained by these methods is often too general and is impaired by false identifications. Therefore, a better understanding of the biological role of miRNAs requires mapping of the exact miRNA-mRNA interactions that occur *in vivo*. *Drosophila melanogaster* provides several unique advantages over other model organisms in the study of miRNA functional targeting. The majority of its miRNAs are evolutionarily conserved up to humans, suggesting that they regulate similar pathways across organisms. Complete genome-wide collections make *Drosophila* the only organism that enables constitutive and inducible gain and loss-of function manipulations of all annotated miRNAs. These powerful tools led to several groundbreaking discoveries of the role that miRNAs play in regulation of development, stem-cell function and aging, and proved that although many outcomes are possible, most *Drosophila* miRNAs regulate a single phenotype through downregulation of a single major mRNA target.

KEY WORDS: *microRNAs, Drosophila, miRNA-sponge, miRNA-sensor, mRNA targets*

Introduction

As their name implies, microRNAs (miRNAs) are small RNA molecules ranging between 18-26 nucleotides. Like messenger RNAs (mRNAs), miRNAs are encoded in the organism genome of eukaryotic cells, transcribed by RNA polymerase II, processed at the nucleus and exported to the cytoplasm. At the cytoplasm, their function is to uncouple targeted mRNAs from the translation machinery and thus prevent their translation into active proteins (Carthew *et al.*, 2016, Ghildiyal and Zamore, 2009). Fortunately, the answer for the intriguing question of why should the cell transcribe an mRNA, process it and export it into the cytoplasm only to be degraded by a miRNA, came immediately with the discovery of miRNAs in *C. elegans*. The function of the first miRNAs identified, *lin-14* and *let-7* (Lee and Ambros, 2001, Lee *et al.*, 1993, Wightman *et al.*, 1993), is to clear out the transcription program that defines the previous developmental stage and by that enable developmental progression. Further research has shown that miRNAs provide a strong regulatory network which allows cells to quickly transition from one stage to the other, as well as to allow the whole organism to change course during embryonic development, adulthood and aging (Carthew *et al.*, 2016, Garg and Cohen, 2014). miRNAs can regulate many types of transitions, such as stem cells to differentiated progeny, dynamic adherence between cells or internal regulation of the cell cycle (Ma *et al.*, 2010, Melton *et al.*, 2010, Yu *et al.*, 2009). However, miRNAs also dictate stable cellular properties like fate commitment or protection from apoptosis. In this latter role, antiapoptotic miRNAs act as survival factors by blocking the expression of proapoptotic genes and provide a unique defense mechanism from apoptotic events (Brennecke *et*

Abbreviations used in this paper: miRNA, microRNA.

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Accepted: 10 August 2016.

ISSN: Online 1696-3547, Print 0214-6282

al., 2003, Carthew *et al.*, 2016).

A miRNA regulates its mRNA targets through binding to a small primary recognition sequence of 6-8 nucleotides. These regulatory sequences are located throughout the mRNA but their presence is predominantly in the 3' untranslated region (3'UTR). The 6 nucleotides seed sequence is located at the 5' of the miRNA, between nucleotides 2-8. This seed recognizes and base-pair with the anti-sense recognition sequence of the mRNA (Ghildiyal and Zamore, 2009). Because this type of recognition is obligatory, mRNA targets for each miRNA can be bioinformatically predicted. Indeed several hundreds to thousands mRNAs are predicted targets for each miRNA (Shin *et al.*, 2010). miRNA target prediction also takes into account the free energy of miRNA and mRNA interactions and mRNA recognition site conservation throughout evolution (Enright *et al.*, 2003, Lewis *et al.*, 2005). While some targets were experimentally confirmed, most of the predicted targets are yet to be validated. The use of bioinformatics often leads to false-positive identification, as experimentally proven using a shuffled miRNA sequence (Lewis *et al.*, 2003). Therefore, not every mRNA that contains the seed sequence is indeed a direct target of the corresponding miRNA. Furthermore, even when a mRNA is a possible target for a certain miRNA, both should be adequately expressed in the same cell at the same time for repression to occur, which is not always the case.

Some studies using molecular biology techniques suggest that a single miRNA downregulates many mRNA targets simultaneously. This was shown in mammalian models where, for example, *mir-1* and *mir-124* that are mainly expressed in the muscle and brain were found to downregulate 96 and 174 genes, respectively. However, these experiments were done by over-expression of the selected miRNA in cells that do not normally express such high levels (Lim *et al.*, 2005).

Therefore, one of the general questions of miRNA biology is whether in a given spatial and temporal state, a miRNA targets many mRNAs, as may be suggested by computational and molecular biology studies, or rather represses a single crucial target. Current genetic studies in *Drosophila melanogaster* reveal that individual targets/pathways are often major effectors of miRNA-mediated phenotypes (Bejarano *et al.*, 2012). In this review we will highlight the unique advantages of miRNA research in *Drosophila* and present selected examples to emphasize the strength of model organisms such as *Drosophila* to help distinguish whether "functional targeting" of individual targets are rare or actually common.

The unique toolbox of *Drosophila* for miRNA research

About 140 miRNAs were annotated in *Drosophila* with high confidence, from which 78 display ≥70% sequence similarity to human orthologous (Kozomara and Griffiths-Jones, 2011). *Drosophila* offers several unique advantages in the study of miRNAs as detailed herein.

The biggest advantage is the availability of genomewide collections of inducible loss of function (LOF) and gain of function (GOF) individual miRNAs (Bejarano *et al.*, 2012, Fulga *et al.*, 2015, Schertel *et al.*, 2012). Three of these recently generated collections include Upstream Activating Sequence (UAS) to enable cell specific GOF or LOF of annotated miRNAs in desired tissues with the appropriate GAL4 drivers. The bipartite UAS-GAL4 system enables selective spatial expression of miRNAs from the above collections whereas

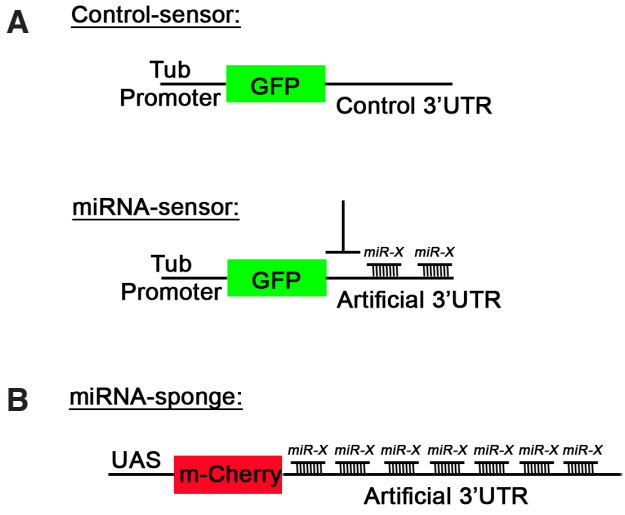

Fig. 1. Schematic representation of miRNA-sensor, control-sensor and miRNA-sponge. (A) *miRNA- and control- sensors are driven by a ubiquitous promoter (*e.g. *Tubulin) and contain a reporter (*e.g. *GFP) coding region. The miRNA-sensor contains an artificial 3'UTR with 2-3 repeats of antisense sequences towards a given miRNA (*mir-X*), whereas the control-sensor contains a control 3'UTR (Brennecke* et al.*, 2003, Schertel* et al.*, 2012).* **(B)**. *An miRNA-sponge contains a UAS element to drive spatial restricted expression with an appropriate GAL4 driver, followed by a reporter (*e.g. *m-Cherry) coding region. An artificial 3'UTR containing many repeats of antisense sequences towards a given miRNA (*mir-X*) is used as a decoy to deplete* mir-X *from a desired cell population (Fulga* et al.*, 2015, Herranz* et al.*, 2012).*

otherwise overall loss or gain expression may be lethal (Phelps and Brand, 1998). Moreover, a temperature-sensitive allele of the GAL80^{ts} repressor enables conditional expression restricted to a certain developmental stage or during adulthood thus providing spatially and temporally restricted expression (Suster *et al.*, 2004).

The LOF sponge collection includes *UAS-mCherry-miRNA-sponge* transgenes that permit conditional miRNA inactivation in specific cells (Fig. 1B). Each sponge transgene includes an artificial 3'UTR region following mCherry coding sequence. The 3'UTR region contains 20 binding sites for a given miRNA that are anti-sense sequences that act as a decoy to dilute miRNA expression (Ebert and Sharp, 2010, Fulga *et al.*, 2015). The collection also includes mCherry to validate transgene expression.

The two GOF miRNA transgene collections that were recently generated cover the entire repertoire of *Drosophila* miRNAs and offer a useful tool to complement the LOF approaches (Bejarano *et al.*, 2012, Schertel *et al.*, 2012). Inducible GOF expression also circumvents redundancy difficulties of miRNAs for which LOF conditions do not yield a distinct phenotype. In one of the GOF collections, the transgenes are marked by DsRed to enable *in-vivo* tracking of miRNA overexpression (Bejarano *et al.*, 2012). The joint effect of the GOF phenotype and its LOF counterpart, along with their coverage of each individual miRNA enables their use for genetic screens, individual miRNA research and identification of synergies between signaling pathways and miRNAs. So far these collections revealed hundreds of distinct phenotypes illustrating the power of genetics in identifying potent and unexpected functions of miRNAs that cannot be predicted by computational approaches and

molecular biology studies *(Bejarano et al., 2012, Fulga et al., 2015, Schertel et al., 2012). Drosophila* is currently the only species for which such collections of inducible miRNA transgenic tools exist.

In addition to the inducible collections, genomewide null-miRNA mutations were obtained by targeted approaches, collectively deleting 99% of *Drosophila* miRNAs (Chen *et al*., 2014). A similar genomewide collection was previously obtained in *C. elegans* in which mutations in the majority of miRNAs did not result in loss of viability or abnormal developmental phenotypes, probably due to functional redundancy (Miska *et al*., 2007). However, when tested in genetically sensitized backgrounds, ~80% of *C. elegans* miRNAs showed mutant phenotypes, supporting the notion that redundant function exists to ensure robustness of developmental processes (Brenner *et al*., 2010). In contrast to the *C. elegans* null collection, 80% of *Drosophila* miRNA null mutants exhibited significant phenotypes in normal background, at least in one of the following assays: survival, fertility, lifespan, primordial germ cell (PGC) number, external morphology (*e.g.* eye or wing defects), climbing behavior or hemolymph-brain barrier (Chen *et al*., 2014, Miska *et al*., 2007). This makes *Drosophila* a preferable model and suggests that the null collection will be invaluable for any additional *in vivo* functional analysis of miRNAs (Chen *et al*., 2014, Miska *et al*., 2007).

Another important method that is often used for miRNA research in *Drosophila* is the miRNA-sensor (Fig. 1A). As miRNAs are not immunogenic and antibodies cannot be created and used to identify their expression, miRNA-sensors were developed to *in vivo* monitor the expression pattern of a given miRNA. Similarly to the previously described miRNA-sponges, miRNA-sensors use the ability of miRNAs to silence the expression of their specific targets for *in-vivo* detection. A miRNA-sensor is comprised of few of the complementary sequences of the selected miRNA (usually two-three repeats) within an artificial 3'UTR region of a reporter GFP sequence. If a certain miRNA is present in a given cell population, it activates the silencing mechanism leading to disappearance of the GFP signal. Expression of the transgene is driven by a ubiquitous promoter (*e.g.* Tubulin) and the GFP levels are compared to those observed in unaffected cells. A control sensor carrying a control 3'UTR should be tested as well to verify that the pattern of GFP expression does not merely reflect the pattern of the driver expression (Fig. 1). Furthermore, testing the sensors in respective mutants should reveal their reliability. Currently, sensors exist for only a small fraction of *Drosophila* miRNAs but creating a GFP sensor transgene to characterize a given miRNA expression and test for direct genetic interactions *in vivo* is relatively simple to perform (Brennecke *et al*., 2003, Schertel *et al*., 2012).

The last advantage we would like to review here is the existence of two Dicer RNase III enzymes in *Drosophila*, Dicer-1 and Dicer-2. Dicer is a key processor of small RNAs that acts at the cytoplasm during the final stages of their biogenesis (Ghildiyal and Zamore, 2009). In contrast to other model organisms (*e.g. C. elegans* and mice) that have one Dicer enzyme to process both miRNAs and small interfering RNAs (siRNAs), in *Drosophila* Dicer-1 is devoted to miRNA processing whereas Dicer-2 generates siRNAs (Lee *et al*., 2004, Miyoshi *et al*., 2010). This segregation enabled further identification of components that selectively regulate miRNA biogenesis (Siomi and Siomi, 2010). Moreover the segregation generates a unique opportunity to use to RNAi machinery as a tool to reduce *Dicer-1* in a desired cell population and thus to remove miRNA production in these cells only. This approach can be used in post-mitotic adult tissues as *Dicer-1* null mutants are lethal during embryonic development and germline clones are not possible in non-dividing cells. Removing miRNA production selectively from a group of cells *in vivo* may provide a means to identify critical and unique functions of miRNAs.

Drosophila miRNAs downregulate distinct major targets

In this part we focus on a small fraction of the miRNA research done in *Drosophila* using the unique tools described above. All of these examples prove that at a certain place and time a single mRNA target is responsible for a unique miRNA phenotype (Fig. 2).

bantam (*ban*) is one of the most critical miRNAs for *Drosophila* development and its null mutants exhibit significantly reduce viability (Brennecke *et al*., 2003, Chen *et al*., 2014). GOF studies have shown that *ban* induces cell growth and a GFP-*ban* sensor revealed a striking correlation between its expression pattern and cell proliferation (Brennecke *et al*., 2003). Moreover, a *ban*-sponge used to deplete *ban* expression in selected dorsal compartment of the wing imaginal disc resulted in reduced tissue growth (Herranz *et al*., 2012). Co-expression of the *ban*-sensor and the *ban* sponge led to higher levels of sensor GFP levels proving the efficiency of the *ban*-sponge in depleting *ban in vivo* (Fig. 1). Consistent with its role in cell growth, *ban* is transcriptionally activated downstream of the Hippo-Yorkie, BMP and EGFR growth control signaling pathways that determine cell proliferation and organ size (Herranz *et al*., 2012, Nolo *et al*., 2006, Oh and Irvine, 2011, Thompson and Cohen, 2006). *ban* positively affects tissue growth by suppressing negative growth regulators, one being the pro-apoptotic IAP antagonist, Hid (Chen *et al*., 2014). The mRNA of the proapoptotic *hid* possesses five recognition elements for *ban* seed in its 3'UTR and *hid* was characterized as a bona-fide target for *ban* (Brennecke *et al*., 2003). Thus, *ban* can provide the cells protection from apoptosis and enable cell proliferation during development because it acts downstream of many apoptotic signals at the execution level of Hid expression. In the ovary of adult females *ban* provides selective protection from apoptosis only to the germline stem cell (GSC) population by blocking translation of the same target, *hid*. Upon apoptosis induction by genotoxic signals such as irradiation, the stem cells remain intact while the neighboring differentiated cells undergo apoptosis (Xing *et al*., 2015). Although there is no *ban* orthologue in mammals, these studies paved the way for identification of antiapoptotic miRNAs and their role in human diseases including cancer (Lima *et al*., 2011). *ban* is also expressed in another stem cell population, the larval brain neuroblasts while it is absent from differentiated progeny as revealed by *ban* GFP-sensor. In neuroblasts *ban* downregulates the differentiation factors *prospero* and *brat* to enable stem cell maintenance (Weng and Cohen, 2015). Another critical cellular function of *ban* is to regulate the circadian rhythm. Circadian clocks act autonomously in all cells and are generated by feedback loops of interconnected transcription factors including Clock that is present in limiting amounts to ensure adaptation to daily environmental cycles. GOF of *clock* that carries mutations in the recognition elements for *ban* and is thus resilient for post-transcriptional regulation, resulted in defects in circadian neurons development (Lerner *et al*., 2015). These examples reveal that *ban* miRNA has diverse functions in different time, cellular and tissue contexts, each mediated by downregulation of one

key mRNA target.

Another multifunctional miRNA in *Drosophila* is *miR-9a*. Unlike *ban*, the *miR-9a* nucleotide sequence is evolutionarily conserved between *Drosophila*, mice and humans. Moreover, *miR-9a* mutants are viable, fertile and reach adulthood (Li *et al.*, 2006). *miR-9a* is a clear example whereby an easily distinguished LOF morphological phenotype can be mimicked by manipulating the recognition site of a single mRNA target of the *Drosophila LIM-Only (dLMO)* gene. dLMO serves as a transcription cofactor which inhibits Apterous directly (Milan *et al.*, 1998). Apterous is required for the integrity of the *Drosophila* wings on the dorsal side. When *miR-9a* was deleted from the genome, a unique no-margin wing phenotype occurred. A similar phenotype was obtained in a separate experiment in which the 3'UTR of *dLMO* was removed. These two identical phenotypes that emerged inconsequently to each other suggest that there is a strong and exclusive link between *miR-9a* and its target, *dLMO* (Biryukova *et al.*, 2009). This phenotype was a result of apoptosis, and was completely reversed when a single allele of *dLMO* was removed, thus inhibiting its endogenous expression, regardless of the presence of *miR-9a*. Moreover, GOF of UAS-DsRed:*miR-9a* in the pouch domain of the wing imaginal disc resulted in specific reduction of GFP-dLMO sensor, implying a direct regulation of *miR-9a* via *dLMO* 3'UTR (Bejarano *et al.*, 2010).The *miR-9a* recognition element is conserved in the human orthologue of the T-cell acute leukemia oncogene (LMO2), suggesting that *miR-9* may also downregulates LMO2 expression under harmful levels (Biryukova *et al.*, 2009).

miR-9a mutants exhibit additional morphological phenotypes: ectopic formation of sensory neurons in embryos and excessive number of sensory organs in adults. *miR-9a* is expressed in epithelial cells prior to their specification as SOP and downregulates the expression of the transcription factor *senseless* thereby inhibiting the SOP fate in the surrounding epithelial cells and limit the number of SOP (Carthew *et al.*, 2016, Li *et al.*, 2006). The interaction between *miR-9a* and its target *senseless* also helps to ensure developmental robustness during SOP specification as *miR-9a* mutants show high variability among individuals in the number of sensory organs. This variability increases when *miR-9a* mutants are exposed to environmental and/or genetic variations (Cassidy *et al.*, 2013).

miR-9a has another role in a different time and place. During embryonic development, it regulates the expression of Dystroglycan (Dg), an extracellular matrix (ECM) receptor necessary for the formation of myo-tendinous junctions. Muscles express Dg while their neighboring tendons express *miR-9a* which down regulates Dg. This allows correct formation of muscle-tendon connections. *miR-9a* serves as a "bouncer" selectively blocking expression of muscle factors in tendons and thus protecting their specific cell fate. (Yatsenko & Shcherbata, 2014).

In the *Drosophila* class I dendrite arborization (da) neurons, *miR-9a* attenuates dendrite development by suppressing the cadherin protein Flamingo (Fmi). *miR-9a* lacks from the neurons and expresses exclusively in the epithelium thus the expression of epithelial but not neuronal Fmi is downregulated. Fmi forms homophilic adhesion interactions and influences axonal growth and navigation by affecting the adhesion between primary and following axons. In dendrite growth, Fmi plays a repressive role and in the absence of *miR-9a,* the excess Fmi binds to neuronal Fmi and causes unnecessary dendrite growth (Wang *et al.*, 2016). Interestingly, in mammals *miR-9* also regulates adhesion by downregulating another cadherin molecule, *Epithelial-cadherin. miR-9a* is upregulated in breast cancer leading to increased cell motility and invasiveness (Ma *et al.*, 2010).

Another proven target of *miR-9a* is the short neuropeptide receptor F1 (sNPFR1), a modulator of insulin signaling. The Insulin/IGF pathway regulates body growth in *Drosophila*, through the neurosecratory insulin-producing cells located in the fly brain. The cells produce three insulin-like peptides (*Dilps*) among which, *Dilp2* is the dominant one controlling body growth. *Dilp2* expression is regulated by the connection between sNPF and its receptor, sNPFR1. When overexpressed in the insulin-producing cells of the brain, *miR-9a* inhibits the expression of sNPFR1, which in turn leads to reduced body size, wing length and pupal volume. When *miR-9a* is knocked-down, the phenotype is reversed to an increased body size and wing length, as well as larger pupal volume (Suh *et al.*, 2015).

miR-9a mutant flies also have an aging phenotype of a short lifespan. It will be interesting to identify the mRNA targets whose downregulation is essential for a normal lifespan (Chen *et al.*, 2014).Three additional miRNAs where found to regulate aging related phenotypes: *let-7*, *miR-34* and *miR-1000 (Liu et al., 2012, Toledano et al., 2012, Verma et al., 2015)*. *let-7* was initially described as a regulator of the developmental timing pathway, namely the *heterochronic* pathway (Ambros, 2011). *let-7* regulation of stem cells during aging was first described for neuronal stem cells in the mouse forebrain, expending the *heterochronic* function towards adulthood and aging (Nishino *et al.*, 2008,

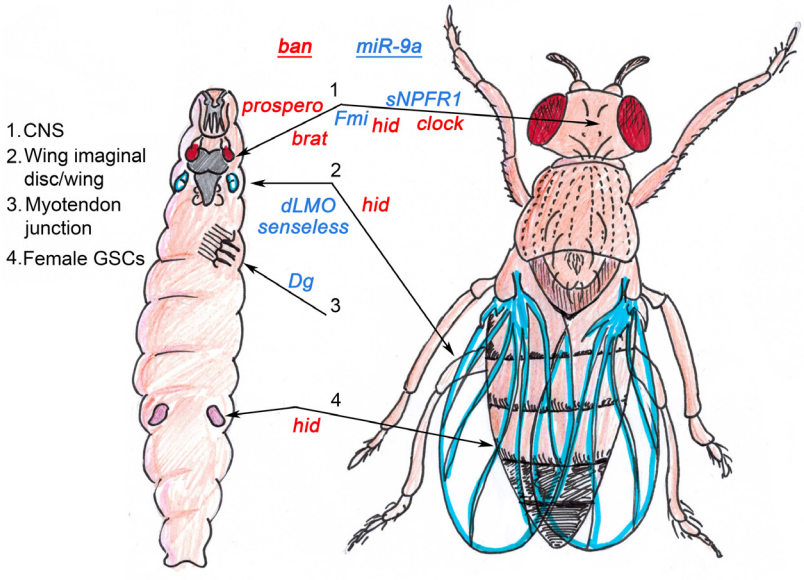

Fig. 2. *ban* and *miR-9a* downregulate direct individual targets in distinct developmental stages and cells. ban *(red) regulates* hid *(red) in the wing imaginal disc, brain hemisphere and adult female GSCs.* ban *also regulates* clock *(red) in the brain,* prospero *(red) and* brat *(red) in neuroblasts.* miR-9a *(blue) regulates sNPFR1 in adult brain and Fmi in larvae CNS.* miR-9a *also regulates* dLMO *and* senseless *in the wing imaginal disc and* Dg *in the tendons.*

Toledano, 2013). In the *Drosphila* male (germline stem cell) GSC niche, *let-7* is expressed in the hub cells that represent a major component of the somatic niche. We demonstrated that elevated levels of *let-7* in the hub of aged males initiate a cascade of events that culminates in decreased niche function and GSCs loss. The increase in the levels of *let-7* directly regulates the mRNA of the evolutionarily conserved RNA binding protein *imp*, and reduces self-renewal function of the niche (Toledano *et al*., 2012).

miR-34 and *miR-1000* deficient animals have a shortened lifespan and they exhibit early-onset neurodegeneration (Liu *et al*., 2012, Verma *et al*., 2015). Furthermore, they both exhibit brain-enriched and age-modulated characteristics. However, while the levels of *miR-34* increase in the brain during aging, the levels of *miR-1000* decreases. *Eip74EF* (E74A) was characterized as a *miR-34* target and elevated levels of E74A were found in the *miR-34* mutant to be responsible for the degeneration and short lifespan (Liu *et al*., 2012).

miR-1000 is expressed in the *Drosophila* CNS throughout the life of the fly, its target being the vesicular glutamate transporter (*VGlut*). VGlut is responsible for loading glutamate onto synaptic vesicles to be released at the synaptic junction. When *miR-1000* was deleted from the genome, there was an overexpression of VGlut which resulted in excessive glutamate release leading to fly death due to excessive stimulation of nerve cells. When rescued through reintroduction of *miR-1000*, the flies survived at a rate similar to the control (Verma *et al*., 2015). This shows a tight connection between one miRNA and one mRNA target, in a specific tissue that affect the overall organism lifespan.

Concluding remarks

Our knowledge of stages in animal development relies on tools and techniques designed to detect cellular events that occur within the whole organism. In the past, *Drosophila was* mainly used to perform forward- and reverse genetic screens. Currently, the development of advanced biochemical and molecular biology techniques has enabled the use of *Drosophila* to manipulate gene expression in small specialized cell populations. In the miRNA field, *Drosophila* became an attractive model to study dynamic interplays between miRNA and mRNA targets. It enables us to specifically alter one small binding sequence of miRNA-mRNA and to study the effect at multiple levels of cellular and whole organism function. In recent years, miRNA research in *Drosophila* largely contributed to the understanding that this class of post-transcriptional regulators has a major role in gene expression throughout all cycles of the lifespan.

In this review, we discuss the use of unique tools established for miRNA research in *Drosophila* and show that miRNA at a certain cellular and temporal event regulate only one key target (Fig. 2). This perspective counteracts computational approaches that yielded hundreds to thousands direct target predictions per a single animal miRNA. These predictions were supported by systematic profiling of the transcriptome and suggested that miRNAs have a broad and subtle “fine-tuning” type of regulation.

miRNA research in *Drosophila* emphasizes the gap between bioinformatics and phenotype characterization of GOF and LOF transgenes in model organism. However, at this stage we cannot rule this notion on higher organisms and further research in mammals will confirm whether we can generalize that at the right place and time miRNAs regulate single crucial targets in all animals.

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