Functions of microRNAs in *Drosophila* development

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Abstract

Control of mRNA translation and degradation has been shown to be key in the development of complex organisms. The core mRNA degradation machinery is highly conserved in eukaryotes and relies on processive degradation enzymes gaining access to the mRNA. Control of mRNA stability in eukaryotes is also intimately linked to the regulation of translation. A key question in the control of mRNA turnover concerns the mechanisms whereby particular mRNAs are specifically degraded in response to cellular factors. Recently, microRNAs have been shown to bind specifically to mRNAs and regulate their expression via repression of translation and/or degradation. To understand the molecular mechanisms during microRNA repression of mRNAs, it is necessary to identify their biologically relevant targets. However, computational methods have so far proved unreliable, therefore verification of biologically important targets at present requires experimental analysis. The present review aims to outline the mechanisms of mRNA degradation and then focus on the role of microRNAs as factors affecting particular *Drosophila* developmental processes via their post-transcriptional effects on mRNA degradation and translation. Examples of experimentally verified targets of microRNAs in *Drosophila* are summarized.

RNA stability and development

During development, the expression of specific proteins is tightly controlled both spatially and temporally to produce a complex multicellular organism capable of reproduction. This requires gene expression to be controlled at each step of the transcriptional and post-transcriptional pathway. Although transcriptional regulation has been widely studied, post-transcriptional processes such as mRNA degradation, and its relationship to protein translation, are less well understood. One way to gain an understanding of the mechanisms of control of gene expression in relation to development is to study a well-characterized model organism, such as *Drosophila melanogaster*. The present review aims to outline the mechanisms of mRNA degradation and then focus on the role of miRNAs (microRNAs) as factors affecting particular *Drosophila* developmental processes via their post-transcriptional effects on mRNA degradation and translation.

The importance of mRNA degradation in development is illustrated by the phenotypes of animals where mRNA turnover pathways have been disrupted. For example, recent work in *Drosophila* has shown that Xrn-1 (known as Pacman) plays a role in epithelial sheet movements, such as dorsal closure, thorax closure, wound healing and fertility [1,2]. Control of mRNA stability is also intimately linked with the regulation of translation. This is again best illustrated in *Drosophila*, where crucial protein gradients are often dependent on translational repression of particular mRNAs followed by their degradation [3]. Translationally repressed mRNAs, and mRNAs targeted for degradation, are often located in P-bodies (processing bodies), which are small cytoplasmic foci containing enzymes of the core 5′→3′ decay machinery [4].

The core eukaryotic mRNA degradation machinery is illustrated in Figure 1. The first step in the mRNA-degradation process is usually cleavage of the 5′ cap (decapping) or removal of the 3′ poly(A) tail (deadenylation), to decircularize the mRNA and allow access to the helicases and degradation enzymes. Deadenylation allows 3′→5′ degradation by the multifunctional exosome complex, whereas decapping allows degradation in the 5′→3′ direction by the exoribonuclease Xrn-1. Alternatively, the mRNA may be cleaved during processes such as RNAi (RNA interference) and the transcript degraded in both directions by 5′→3′ and 3′→5′ exoribonucleases [5].

Specificity of mRNA turnover relies on proteins or other factors specifically targeting the degradation machinery to the mRNA. Typically, factors binding to the 3′-UTR (untranslated region) of a transcript differentially target it for degradation and/or repress its translation. For example, the protein Smaug binds to and causes deadenylation of maternal mRNAs at the maternal–zygotic transition during *Drosophila* embryogenesis [6]. Recently, miRNAs have also been found to bind to mRNAs and specifically affect the expression of their encoded proteins, usually by blocking their translation or promoting transcript deadenylation and turnover. The remainder of the present review concentrates on the mRNA targets and known biological functions of miRNAs in *Drosophila*.

**Key words:** development, *Drosophila*, microRNA (miRNA), mRNA, RNA stability, RNA turnover.

**Abbreviations used:** Ago, Argonaute; dLMO, *Drosophila* LIM-only; DPP, decapentaplegic; mRNA, microRNA; P-body, processing body; PDK, phosphoinositide 3-kinase; RNAi, RNA interference; TCF, T-cell factor; UTR, untranslated region.

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Regulation of gene expression by miRNAs

miRNAs are a family of non-protein coding RNAs that have emerged as important regulators of gene expression. miRNAs are expressed as long-hairpin-forming precursor RNAs which are cleaved into partially double-stranded RNAs and then processed further into mature miRNAs of approx. 22 nucleotides (Figure 2). They are known to associate with Ago (Argonaute) proteins and guide them to target transcripts for their regulation [7]. miRNAs have imperfect complementarity to sequences in the 3′-UTRs of target mRNAs with the ‘seed region’, consisting of nucleotides 2–8, being the most important for determining their mRNA targets. The complementarity of the remaining miRNA sequence is thought to affect the fate of the target mRNA, for either degradation or translational repression [8]. miRNAs bound to Ago1 are found in cytoplasmic P-bodies, suggesting that they can associate with enzymes of the core degradation machinery [9]. The detailed mechanisms of action of miRNAs are at present the subject of intense investigation [10–13].

To understand the molecular mechanisms whereby miRNAs repress their targets, it is necessary to identify their biologically relevant targets. Current computational methods typically find hundreds of potential mRNAs containing binding sites for particular miRNAs, with multiple candidate binding sites per mRNA and little overlap between methods. A typical example is miR-315, which is predicted to bind to 667 sites in 417 mRNAs by TargetScanFly [14], 542 mRNAs by PicTar [15] and 307 mRNAs by Microcosm [16]. However, miR-315 has, as yet, only been experimentally shown to bind to two mRNAs [17]. Although computational methods can be used as a guide, verification of biologically important targets at present requires experimental analysis.

Experimental validation of miRNA targets in Drosophila

One direct way to identify the target(s) of particular miRNAs is to immunoprecipitate mRNAs bound to their targets in cell lysates from cells of interest. In human cells, the PAR-CLIP (photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation) technique, which involves incorporation of photoactivatable nucleoside analogues into RNA, in vivo cross-linking and then immunoprecipitation of Ago1, has proved particularly effective in identifying many hundreds of miRNA and candidate target mRNAs [18]. In Drosophila S2 cells, Ago1 immunoprecipitation of RNA–protein complexes has been shown to detect candidate targets of particular miRNAs (miR-1 and miR-184 [19,20]). However, there is only one case to date where this immunoprecipitation approach has contributed to identifying new miRNA targets which can be verified in vivo in the fruitfly. This study used natural tissues (head lysates) to identify a circadian-regulated miRNA–mRNA
**Figure 2 | Eukaryotic miRNA biosynthesis**

The pri-miRNA (primary miRNA) is typically transcribed from intergenic or intronic regions of the genome by RNA polymerase II and is cleaved in the nucleus by Drosha and its partner DGCR8 (Pasha) to form the pre-miRNA. The 2′-nt overhang at the 3′ end is recognized by Exportin 5 and the pre-miRNA is exported to the cytoplasm, where it undergoes hairpin removal by Dicer-1 to produce the miRNA–miRNA* complex. The mature miRNA is separated from the passenger strand (the miRNA*) and associates with Ago, part of the RISC (RNA-induced silencing complex). In some instances, the miRNA* can also associate with Ago and itself target mRNAs. The ‘seed region’ (nucleotides 2–8) of the miRNA plays the largest part in determining the target mRNAs. Either translational repression or degradation of the mRNA can occur, depending on the level of complementarity between the miRNA and the target.

pair (bantam·clock) [21]. It is possible that binding of miRNAs to their targets may require tissue-specific components which are not present in certain tissue culture cell types.

In general, true biological miRNA targets have been identified in Drosophila using classical genetic techniques, followed by computational analysis to identify candidate mRNA targets. The relevance of these targets in vivo is then usually verified by analysing the expression of a reporter (e.g. luciferase) linked to wild-type or mutated 3′-UTRs in S2 cells. Finally, transgenics expressing mutated versions of the 3′-UTR, together with overexpression of the relevant miRNA, are used to confirm the in vivo significance of the miRNA–mRNA pair (see, e.g., [22]). Examples of the experimental methods used to identify particular miRNA targets together with their known in vivo functions are given below and in Table 1. This Table also lists further experimentally validated miRNA targets known to date in Drosophila.

**miRNA targets and their biological functions in Drosophila melanogaster**

**bantam**

The miRNA bantam was one of the first miRNAs found in Drosophila. It was identified in a gain-of-function screen to detect the genetic element in a 41 kb area of the chromosome (lacking any protein-encoding genes) which caused the bantam (i.e. small) phenotype. Loss of the
### Table 1 | Experimentally verified miRNA–mRNA interactions in *D. melanogaster*

Homology with miRNAs in *Homo sapiens* and *Caenorhabditis elegans* and one or more other *Drosophila* is indicated with Hs, Ce and D respectively, with the name in that species given in parentheses if different from *D. melanogaster*. Homology information was retrieved from miRBase release 15 and corresponding references.

<table>
<thead>
<tr>
<th>Drosophila miRNA</th>
<th>Homologues</th>
<th>Verified mRNA target(s)</th>
<th>Location/processes affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>bantam</td>
<td>D</td>
<td>clock</td>
<td>Circadian rhythm [21]</td>
</tr>
<tr>
<td>bantam</td>
<td>D</td>
<td>hid</td>
<td>Suppression of apoptosis in larvae/imaginal discs [23]</td>
</tr>
<tr>
<td>miR-315</td>
<td>D</td>
<td>Axin, Notum</td>
<td>Up-regulates Wingless pathway in wing imaginal discs [17]</td>
</tr>
<tr>
<td>miR-8</td>
<td>Hs (miR-141 and miR-200), Ce, D</td>
<td>wntless, TCF, CG32767</td>
<td>Repression of Wingless signalling pathway [27]</td>
</tr>
<tr>
<td>miR-8</td>
<td>Hs (miR-141 and miR-200), Ce, D</td>
<td>atrophin</td>
<td>Prevention of apoptosis/neurodegeneration [28]</td>
</tr>
<tr>
<td>miR-8</td>
<td>Hs (miR-141 and miR-200), Ce, D</td>
<td>u-shaped</td>
<td>Insulin signalling and PI3K activity in the larval fat body [29]</td>
</tr>
<tr>
<td>miR-9a</td>
<td>Hs (miR-9), D</td>
<td>dMAG</td>
<td>Apoptosis during wing development [32,33]</td>
</tr>
<tr>
<td>miR-9a</td>
<td>Hs (miR-9), D</td>
<td>senseless</td>
<td>Sensory organ precursor and neuronal precursor cell specification in wing imaginal discs [31]</td>
</tr>
<tr>
<td>miR-184</td>
<td>Hs, D</td>
<td>saxophone</td>
<td>Germline stem cell differentiation [34]</td>
</tr>
<tr>
<td>miR-184</td>
<td>Hs, D</td>
<td>K10</td>
<td>Dorsoventral patterning of the egg shell [34]</td>
</tr>
<tr>
<td>miR-184</td>
<td>Hs, D</td>
<td>tramtrack</td>
<td>Anteroposterior blastoderm patterning [34]</td>
</tr>
<tr>
<td>miR-iab-4-5p</td>
<td>D</td>
<td>Ultrabithorax</td>
<td>Wing/haltere specification [38]</td>
</tr>
<tr>
<td>let-7</td>
<td>Hs, Ce, D</td>
<td>abrupt</td>
<td>Neuromuscular remodelling and temporal organization during metamorphosis [39,40]</td>
</tr>
<tr>
<td>miR-1</td>
<td>Hs, Ce, D</td>
<td>Delta</td>
<td>Notch signalling/cardiac differentiation [41]</td>
</tr>
<tr>
<td>miR-2</td>
<td>Ce, D</td>
<td>reaper, grim, sickle</td>
<td>Apoptosis during embryogenesis [42,43]</td>
</tr>
<tr>
<td>miR-13</td>
<td>Ce, D</td>
<td>reaper, grim, sickle</td>
<td>Apoptosis during embryogenesis [42,43]</td>
</tr>
<tr>
<td>miR-11</td>
<td>D</td>
<td>reaper, grim, sickle</td>
<td>Apoptosis during embryogenesis [42,43]</td>
</tr>
<tr>
<td>miR-6</td>
<td>D</td>
<td>hid</td>
<td>Apoptosis during embryogenesis [42,43]</td>
</tr>
<tr>
<td>miR-308</td>
<td>D</td>
<td>sickle, grim</td>
<td>Apoptosis during embryogenesis [42,43]</td>
</tr>
<tr>
<td>miR-7</td>
<td>Hs, Ce, D</td>
<td>hairy, E(spl)</td>
<td>Notch signalling/wing development [42,44]</td>
</tr>
<tr>
<td>miR-7</td>
<td>Hs, Ce, D</td>
<td>yan</td>
<td>Photoreceptor differentiation [44,45]</td>
</tr>
<tr>
<td>miR-14</td>
<td>D</td>
<td>Ec dysone receptor</td>
<td>Ec dysone signalling/physiology and lifespan [46]</td>
</tr>
<tr>
<td>miR-133</td>
<td>Hs, D</td>
<td>nPTB</td>
<td>Splicing of muscle-specific exons [47]</td>
</tr>
<tr>
<td>miR-278</td>
<td>D</td>
<td>expanded</td>
<td>Insulin signalling [22]</td>
</tr>
<tr>
<td>miR-279</td>
<td>D</td>
<td>nerin-1</td>
<td>Olfactory neuron fate determination [48]</td>
</tr>
</tbody>
</table>

*bantam* locus was shown to result in larvae with slow growth that died as early pupae, often lacking imaginal discs. Induction of expression of the *bantam* locus using P-element insertions resulted in repression of apoptosis and tissue overgrowth, *hid*, a pro-apoptotic gene, was identified computationally as a target of bantam and verified using reporter transcripts with the *hid* 3′-UTR. Co-expression of bantam with *hid* suppressed the apoptotic effect of *hid* in larval wing imaginal discs. In eye imaginal discs, bantam has also been shown to prevent apoptosis caused by induced *hid* expression [23]. More recent work shows that direct up-regulation of *bantam* occurs in uncommitted progenitor cells in eye discs, through the action of the transcription factors Homothorax and Yorkie. When Yorkie is activated by the Hippo pathway, the expression of bantam prevents apoptosis [24,25].

In neuronal development, loss of *bantam* led to overgrowth of dendrites, which would usually only grow in proportion to epithelial cells as the larva grows. Akt was identified as a likely target of *bantam* by mRNA microarrays which showed an increase in its level in neurons and a decrease in epithelial cells in *bantam* mutants. Suppression of the phenotype in these mutants was possible by knocking down Akt with RNAi or by antagonizing Akt.
activity by overexpressing PI3K (phosphoinositide 3-kinase) [26].

*bantam* has also been shown to be involved in circadian behaviours in *Drosophila*. Immunoprecipitation of Ago1 in circadian tissues followed by microarrays was used to identify mRNAs under miRNA control, three of which were core clock mRNAs (*clock, vrille and clockwork orange*). Computational predictions followed by analysis of the clock mRNA 3′-UTR linked to a luciferase reporter construct in *Drosophila* S2 cell culture allowed the identification of three evolutionarily conserved *bantam* sites capable of regulating clock expression [21].

**miR-315**

miR-315 was initially identified in a screen using a TCF (T-cell factor)–luciferase transcription reporter to measure Wingless signalling levels in a *Drosophila* cell culture in which Wg signalling is usually repressed and TCF levels are low. Axin and Notum, negative regulators of the Wingless pathway, were found to be targeted by miR-315 via two predicted sites in each of their 3′-UTRs. These interactions were verified, using luciferase reporters fused to the Axin or Notum 3′-UTRs, by introducing point mutations into each of the seed region sites in the mRNAs, which led to loss of repression by miR-315. Ectopic expression of miR-315 in larval wing imaginal discs resulted in the development of two wing pouches [17].

**miR-8**

miR-8 is another miRNA that has been shown to affect Wingless signalling, but as a repressor rather than an activator. It was identified in a genetic screen designed to detect genes capable of overcoming the small eye phenotype caused by ectopic activation of Wingless signalling in the developing eye. Three mRNA targets were found for miR-8: *wntless*, TCF and a Wingless signalling activator, CG32767. The effect on TCF was shown to be by translational repression rather than mRNA degradation, as protein levels were reduced while mRNA levels remained the same [27].

A mutational approach has identified a separate role for miR-8 in the *Drosophila* nervous system. Deletion of miR-8 by imprecise P-element excision or homologous recombination resulted in fruitflies with leg defects and a lower survival rate compared with that of wild-type. Microarray analysis of pupae detected 200 genes that were reproducibly up-regulated 2-fold or more, four of which had 3′-UTR miR-8 target sites. *atrophin* had four miR-8 sites, two of which are conserved in the 3′-UTR of the human orthologue RERE. Loss of miR-8 was shown to increase the level of *atrophin* in the brain of third instar larvae, leading to an increase in apoptosis in the nervous system [28].

miR-8 has also been shown to alter the body size of adults by affecting insulin signalling in the larval fat body. miR-8 mutants have a reduced adult size, which can be rescued by fat-body-specific miR-8 expression. *u-shaped* was shown to be the relevant target of miR-8, with the level of *u-shaped* mRNA increasing 2-fold and the level of protein increasing further in the mutants. Increased *u-shaped* led to repression of insulin signalling and reduced PI3K activity. The interaction is conserved in humans, where the miR-8 homologue miR-200 targets the human *u-shaped* homologue FOG2 [29].

**miR-9a**

miR-9a is entirely conserved in many species including humans (where it is named miR-9), with the mouse homologue showing brain-specific expression [30]. Loss of miR-9a in *Drosophila* produces a phenotype in adults characterized by an increase in the number of sensory bristles on the anterior wing margins and notum, and a loss of tissue from the posterior wing margin. Overexpression of miR-9a has the opposite effect, reducing the number of sensory bristles. Three target sites for miR-9a were identified computationally in *senseless*, mutants of which display related phenotypes. Genetic interaction between miR-9a and *senseless* was observed as a reduction in *senseless* expression in miR-9a mutants which consequently reduced the loss of posterior wing tissue. *In situ* hybridization and immunostaining experiments on wing imaginal discs showed that miR-9a and *senseless* were expressed in mutually exclusive locations, with a decrease in *senseless* expression when miR-9a was overexpressed [31].

More recently, another target responsible for the loss of posterior wing tissue in miR-9a-null flies has been identified. Loss of miR-9a, or removal of the 3′-UTR of dLMO (*Drosophila* LIM-only), which contains a miR-9a target site, led to apoptosis in the dorsal wing primordium and the corresponding significant loss of adult wing tissue. dLMO was also found to contribute to the bristle phenotype described previously [32,33].

**miR-184**

A number of roles for miR-184 have been identified in the female germline [34]. Fruitflies lacking *miR-184* appear to develop normally, but females lay abnormal eggs and rapidly become infertile. The earliest effect is observed in the ovaries, where the daughters of *miR-184* mutant germline stem cells are prevented from differentiating. Differentiation usually occurs as the daughter cells move away from the niche and express Bag-of-Marbles to become cystoblasts. Within the niche, Bag-of-Marbles expression is prevented by DPP (decapentaplegic) [35]. miR-184 targets a site in the 3′-UTR of the DPP receptor *saxophone*, therefore loss of miR-184 leads to increased and mislocalized Saxophone protein expression, preventing differentiation.

Later during oogenesis, loss of *miR-184* indirectly affects the localization of Gurken protein in the anteriodorsal corner of the oocyte, which is required to cause dorsalization of the nearby follicle cells [36]. *miR-184* affects Gurken localization by acting earlier in development via K10, a nuclear protein required for export of *gurken* mRNA and the only known *gurken* regulator that contains a *miR-184* site in the 3′-UTR of its mRNA. Loss of *miR-184* leads to premature overexpression of K10, followed by a 50% reduction in K10 activity by overexpressing PI3K (phosphoinositide 3-kinase) [26].
protein levels at its normal expression time. This results in almost all eggs becoming dorsIALIZED.

Of the fertilized and morphologically normal eggs, embryos showed delayed expression of pair-rule genes, such as fushi tarazu and odd-skipped, and disruption to runt expression. This was due to overexpression of tramtrack, which is normally down-regulated by miR-184 via a target site in its 3′-UTR. Tramtrack transcriptionally represses fushi tarazu and its overexpression leads to severe disruption of pair-rule genes [37]. The lack of maternally provided tramtrack led to a 2.5-fold increase in expression of Tramtrack protein, which can be partially rescued by using a tramtrack mutant to reduce the maternal contribution of tramtrack mRNA.

Conclusions

The examples above, and in Table 1, illustrate a number of miRNA targets that have been identified in vivo where the miRNA–mRNA interactions have developmental consequences. These validated miRNA interactions provide a basis upon which the molecular mechanisms underlying translational repression or target degradation can be analysed in a tissue-specific context. Understanding these tissue-specific interactions may also help to improve computational predictions of miRNA-binding sites. Furthermore, analysis of miRNA–mRNA interactions in vivo will help us to understand the ways in which miRNAs, together with mRNA turnover and translation control, function in a developmental context within the vast network of interactions in cells and within organisms.

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