# The roles of miRNAs in wing imaginal disc development in *Drosophila*

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## Abstract

**During development, it is essential for gene expression to occur in a very precise spatial and temporal manner. There are many levels at which regulation of gene expression can occur, and recent evidence demonstrates the importance of mRNA stability in governing the amount of mRNA that can be translated into functional protein. One of the most important discoveries in this field has been miRNAs (microRNAs) and their function in targeting specific mRNAs for repression. The wing imaginal discs of *Drosophila* are an excellent model system to study the roles of miRNAs during development and illustrate their importance in gene regulation. This review aims at discussing the developmental processes where control of gene expression by miRNAs is required, together with the known mechanisms of this regulation. These developmental processes include Hox gene regulation, developmental timing, growth control, specification of SOPs (sensory organ precursors) and the regulation of signalling pathways.**

## miRNAs (microRNAs)

miRNAs are endogenous small non-protein coding RNAs of ~22 nt in length that have emerged as important regulators of post-transcriptional gene regulation. They were first discovered in *Caenorhabditis elegans* in 1993 [1], when the gene known to control developmental timing, *let-7*, was found not to encode a protein, but instead a pair of small RNAs [2]. The role of miRNAs is to post-transcriptionally repress target mRNAs. This can either be by cleaving the mRNA, allowing degradation to occur or through repression of translation. It is generally accepted that the fate of the mRNA is dependent on the extent of complementarity between the miRNA seed region (nucleotides 2–8) and the mRNA target site [normally within its 3′-UTR (3′-untranslated region)]. High complementarity is associated with cleavage and low complementarity with translational repression. Animals tend to favour translational repression, whereas cleavage appears to be more common in plants [3–5]. The mechanisms involved in the biogenesis of miRNAs have been extensively reviewed in [6].

Since their discovery, the identification of new miRNAs and their targets, and the wide variety of functions assigned to them has become a vast area of research. This is evident by huge numbers of predicted miRNAs to date, with release 18 of miRBase predicting 21 643 mature miRNAs in 168 species. It is then perhaps no surprise that miRNAs have been identified to have roles in many aspects of development [7,8] and disease [9]. It is important when studying the roles of miRNAs in development that whole organisms are used, as miRNAs are capable of operating very tissue-specifically. With the wide variety of genetic tools available in *Drosophila*, the wing imaginal discs have become an important system to study patterning and growth during development, leading to a detailed knowledge of the mechanisms involved in the formation of the adult wing. Imaginal discs therefore provide an ideal tissue in which to study the roles of miRNAs in development. This review will discuss these various roles and their implications in a wider context, such as the regulation of growth and patterning during development and cancer.

## Wing imaginal disc development

The imaginal discs of *Drosophila* are first specified in the embryo and eventually form all the adult structures of the fly, such as the wings, halteres and legs. The wing imaginal discs grow from roughly 50 to 50 000 cells during larval development and differentiate using a series of complex signalling mechanisms to regulate patterning and growth [10]. During early larval stages, AP (anterior/posterior) and DV (dorsal/ventral) compartments are specified (Figure 1). The dorsal compartment is established by localized expression of Apterous, which specifies dorsal cell fate, whereas the posterior compartment is specified by the localized expression of Engrailed. The ventral and anterior compartments are established by the absence of Apterous and Engrailed respectively. Cells in the posterior compartment communicate with anterior cells through expression of the secreted signalling molecule Hedgehog which leads to localized expression of the secreted signalling molecule Dpp in anterior cells near to the compartment boundary of the wing disc. Localized expression of Dpp is thought to specify cell fates and control growth in the developing wing imaginal discs

**Key words:** *Drosophila*, microRNA (miRNA), sensory organ precursor, signalling pathway, *Ultrabithorax*, wing imaginal disc development.

**Abbreviations used:** *abd-A*, *abdominal A*; AP, anterior/posterior; *dLMO*, *Drosophila LIM-only*; DV, dorsal/ventral; miRNA, microRNA; SOP, sensory organ precursor; *Ubx*, *Ultrabithorax*; 3′-UTR, 3′-untranslated region; ZNC, zone of non-proliferating cells.

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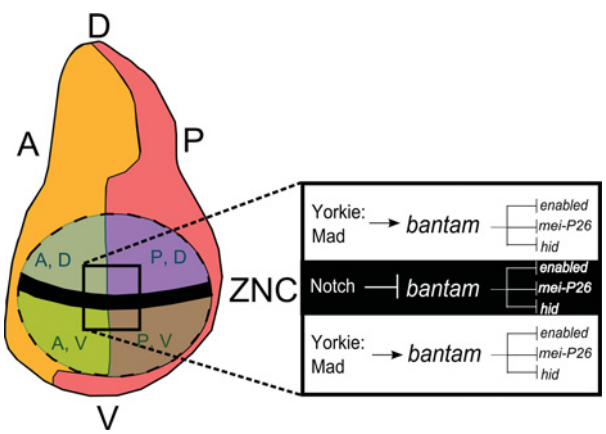
**Figure 1 | Fate map of the third instar wing imaginal disc in *Drosophila* illustrating the role of *bantam* miRNA**

The wing imaginal disc is divided into four compartments by cells which will ultimately form the AP boundary and DV boundary of the wing. The wing pouch, which will develop into the adult wing, is marked by the broken lines. The remainder of the dorsal half of the wing disc will form the part of the thorax and the hinge region. In the ZNC, at the DV boundary, *bantam* is down-regulated as a result of Notch signalling, which contributes to the maintenance of the ZNC through increased levels of *mei-P26*, *hid* and *enabled* [25]. Throughout the remainder of the imaginal disc *bantam* expression is up-regulated through the activity of Yorkie and Mad, contributing to increased growth and proliferation of these cells [27].



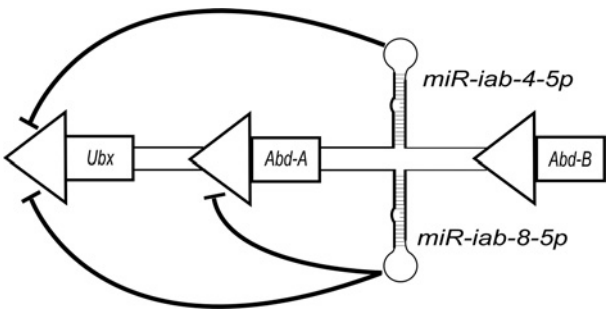

[11]. Interactions between dorsal and ventral cells leads to expression of the signalling molecule Wingless in a row of cells along the DV boundary. This is the consequence of the activation of the transmembrane receptor Notch by its ligands Serrate and Delta. Wingless then acts as a morphogen by signalling away from the boundary [12–15].

## Hox gene regulation

The *iab-4* locus is part of the Bithorax complex and is thought to contain up to nine homoeotic genes, including three Hox genes, *Ubx* (*Ultrabithorax*), *abd-A* (*abdominal A*) and *abd-B* (*abdominal B*). *iab-4* is expressed in restricted domains along the AP-axis, in a spatiotemporal pattern complementary to that of *Ubx*, implying a possible role in *Ubx* regulation. Ectopic expression of *iab-4* results in a haltere-to-wing homoeotic transformation. *miR-iab-4–5p* has been shown to be responsible for this transformation by directly binding to the 3′-UTR of *Ubx*, inhibiting *Ubx* activity *in vivo* (Figure 2). The *iab-4* locus is analogous in position to *miR-196* in vertebrate Hox clusters, which has been shown to interact with the *Hoxb8* 3′-UTR. Furthermore, the *pre-miR-iab4* hairpin is conserved both in sequence and genomic location in species of mosquito, honeybee and flour beetle, which shared a common ancestor roughly 400 million years ago, indicating a conserved evolutionary mechanism of Hox gene regulation [16]. More recent work has shown that the antisense strand for *miR-iab-4*, termed *miR-iab-8*,

**Figure 2 | Regulation of the *Drosophila* Hox cluster by sense and antisense miRNAs**

*miR-iab-4–5p* is an miRNA that represses the expression of *Ubx*. *miR-iab-8–5p* is processed from the antisense strand of *miR-iab-4* and represses *Ubx* and *abd-A*.




also generates a novel pre-miRNA hairpin. *miR-iab-8–5p* (also termed *miR-iab-4AS*) was demonstrated to strongly repress *Ubx* and *abd-A* (Figure 2), with ectopic expression of *miR-iab-8* resulting in lethality as well as nearly complete haltere-to-wing transformations. In addition to identifying a new Hox cluster miRNA, these findings demonstrate that antisense transcription and processing can add a new level of functional diversity to miRNA genes [17,18].

## Developmental timing

Heterochronic genes control the timing of developmental events by forming regulatory networks that temporally regulate cell fates. The *let-7* and *lin-4* miRNAs function as heterochronic switch genes in *C. elegans* by regulating target genes in early and late larval stages respectively [19, 20]. *let-7* is highly conserved and is temporally regulated alongside the *lin-4* homologue *miR-125* in *Drosophila.* It has been demonstrated that mutants devoid of *let-7* and *miR-125* activities show pleiotropic phenotypes during metamorphosis. Two of these phenotypes result from temporal delays in distinct metamorphic processes: the terminal cell cycle exit of cells in the developing wing and maturation of neuromuscular junctions at adult abdominal muscles. The loss of *let-7* was responsible for the cell-cycle defect in the *let-7 miR-125* mutant demonstrating that *let-7* is both necessary and sufficient for the timing of the wing imaginal disc cells to exit the cell cycle approximately 24 h after puparium formation [21]. This is consistent with the role of *let-7* in *C. elegans*, where it is required for the programmed cell-cycle exit of the hypodermal blast cells at the L4-adult transition. This suggests *let-7* has a conserved heterochronic role as a stage-specific timer of cell-cycle exit. The gene *abrupt* has been shown to be an *in vivo* target of *let-7* in pupal wing discs, with the appropriate temporal repression of *abrupt* by *let-7* being required for correct development [21]. Heterochronic genes are considered a major factor that allow for the rapid evolution of new morphologies to take place and the fact that miRNAs are involved in the regulation

of these finely tuned switches, which are required for the development of multi-cellular animals, further emphasizes the importance of miRNAs in an evolutionary context.

## Regulation of cell growth, proliferation and apoptosis

During development, the processes of cell division, cell growth and cell death must be carefully regulated to ensure the correct and proportionate size of the adult organism. Cell proliferation depends not only on signals to stimulate growth and division but also on survival signals that prevent them from undergoing programmed cell death (apoptosis). As mentioned above, the signalling pathways of Hedgehog, wingless/Wnt and Dpp/BMP, which control spatial patterning of the wing imaginal disc are well characterized. Furthermore, the genes involved in cell growth and division of the imaginal disc cells are also well known. However, until recently, little was known how the cells signal to each other to co-ordinate pattern formation with cell proliferation. Brennecke et al. [22] discovered a link between these two mechanisms when they identified the miRNA *bantam* as a pro-survival factor that controls cell proliferation and apoptosis. *bantam* was first identified in a gain of function screen for genes that affect tissue growth [23]. The *bantam* gene identified encodes a 21 nt miRNA that is developmentally regulated and represses the pro-apoptotic gene *hid* by directly binding to its 3′-UTR. Loss of the *bantam* gene resulted in reduced larval growth, pupal lethality and loss of imaginal discs, whereas increased *bantam* expression resulted in repression of apoptosis and tissue overgrowth. To determine the spatial pattern of *bantam* in the third instar wing imaginal discs, a *bantam* sensor transgene was used. *bantam* expression was shown to occur throughout the wing imaginal disc, but was reduced in cells at the AP and DV boundaries including the ZNC (zone of non-proliferating cells) and in patches in the dorsal thorax. Restoring *bantam* activity in these cells was sufficient to direct cells of the ZNC to enter S phase [23].

The ZNC at the DV boundary is regulated by both wingless and notch activity in a signalling mechanism that involves the down-regulation of *bantam* activity, providing a link between morphogens controlling spatial patterning and the regulation of cell proliferation [24]. Becam et al. [25] identified the actin regulator *enabled* as a new target of *bantam*. They demonstrated that both increased *enabled* activity and reduced proliferation rates contributed to the maintenance of the DV boundary [25] (Figure 1). *mei-P26* has also been demonstrated to be a direct target of *bantam*. Mei-P26 is a homologue of TRIM32 and has been shown to down-regulate the oncogene *myc,* reducing its activity in the developing wing. This observation highlights the role of *bantam* in promoting growth as well as preventing apoptosis. Furthermore, *miR-137* was also shown to down-regulate *mei-P26* in S2 cells [26]. *Bantam* has also been shown to be regulated by the Fat-Hippo and Dpp signalling pathways. Downstream transcription factors in each pathway, Yorkie and Mad, act synergistically to induce *bantam* expression by opposing the repressing activity of the transcriptional repressor Brk [27] (Figure 1).

Another miRNA shown to be involved in preventing apoptosis during wing imaginal disc development is *miR-9a*. Loss of *miR-9a* was shown to cause a substantial loss of wing tissue, caused by ectopic apoptosis in the dorsal wing primordium. This phenotype could be rescued by dorsal-specific inhibition of apoptosis. The transcriptional regulator of wing and neural development, *dLMO* (*Drosophila LIM-only*), was identified as a direct target of *miR-9a*. The de-repression of *dLMO* in the *miR-9a* mutant was shown to be the cause of the ectopic apoptosis [28, 29].

## Specification of SOPs (sensory organ precursors)

Prior to its role in preventing apoptosis in the developing wing, *miR-9a* was first identified to function in the specification of SOPs, which are specified in the ectoderm and generate five different cell types in two or three rounds of asymmetric division. Determination of cell fate requires regulated programmes of gene expression, unequally segregated fate determinants and cell polarity machinery [30]. *miR-9a* is required for the determination of SOPs. Loss of *miR-9a* produced ectopic sensory bristles on the anterior wing margin and the notum and the opposite was observed when *miR-9a* was overexpressed. To achieve this function, it was demonstrated that *miR-9a* directly regulates the expression of *senseless,* through its 3′-UTR, to ensure the correct levels within SOPs and adjacent epithelial cells [31]. Multiple roles of *miR-9a* highlight the versatility of miRNA functions and the importance of the cell-specific nature in which miRNAs function. This is reviewed further in [32].

## Regulation of the Hedgehog signalling pathway

The Hedgehog signalling pathway is required for many developmental processes, including wing development in *Drosophila* and has been linked with the genesis of diverse cancers. Friggi-Grelin et al. [33] showed that *miR-12* and *miR-283* are involved in the regulation of antagonistic components of the Hedgehog signalling pathway in the wing imaginal discs. These two miRNAs are part of the same cluster, which also expresses *miR-304* (but this was shown not to be involved). They were identified in a misregulation screen for new regulators of the Hedgehog pathway. *miR-12* and *miR-283* were shown to regulate the 3′-UTRs of the mRNAs *cos2*, *fu* and *smo* in the wing imaginal discs. However, despite mutants lacking the miRNA cluster showing high levels of lethality before the larval stages (88.3%) no Hedgehog-like phenotypes were observed in the dead embryos or the adult structures of the escaper mutants. The authors proposed that the roles of *miR-12* and *miR-283* are to ‘dampen down’ the

**Table 1 | Experimentally verified roles of miRNAs in the development of the wing imaginal discs in *D. melanogaster***

Homology information was taken from miRBase release 18 and corresponding references. D, Ce and Hs refer to other *Drosophila* species, *C. elegans* and *Homo sapiens* respectively.

| *Drosophila* miRNA | Homologues | Verified mRNA target(s) | Role of miRNA during wing imaginal disc development |
|---|---|---|---|
| *miR-iab-4-5p* | D | *Ultrabithorax* | Wing/haltere specification [16] |
| *miR-iab-8-5p* | D | *Ultrabithorax, abdominal A* | Wing/haltere specification [17,18] |
| *let-7* | D, Ce and Hs | *Abrupt* | Terminal cell cycle exit of cells in the developing wing [21] |
| *bantam* | D | *hid, enabled, mei-P26* | Suppression of apoptosis in larvae/imaginal discs [23]; maintenance of the ZNC [25]; promoting growth [26] |
| *miR-9a* | D, Hs (*miR-9*) | *dLMO, senseless* | Apoptosis during wing development [29]; SOP specification in wing imaginal discs [31] |
| *miR-12* | D | *cos-2, fu, smo* | Regulation of the Hedgehog signalling pathway [33] |
| *miR-283* | D, Hs (*miR-216*) | *cos-2, fu, smo* | Regulation of the Hedgehog signalling pathway [33] |

levels of Hedgehog pathway components to act as a buffer for any stochastic fluctuations in the levels of mRNAs involved in the signalling pathway. However, as Cos2 and Smo down-regulate each other, an increase in Cos2 and Smo in the mutant may not lead to any change overall [33]. It may therefore be that the role of *miR-12* and *miR-283* in regulating Hedgehog signalling is redundant in *Drosophila*. This does not mean to say, however, that these miRNAs do not regulate Hedgehog signalling in other organisms, with a biological significance, and indeed *miR-283* is conserved among humans (*miR-216*) (Table 1).

## Using wing imaginal discs to study miRNA/mRNA interactions *in vivo*

In addition to identifying the targets and functions of miRNAs during development, the wing imaginal disc also provides an ideal mechanism for experimentally validating predicted miRNA target sites *in vivo*. For example Stark et al. [34] verified that *miR-7* regulates notch targets *HLMm3*, *m4* and *hairy* [34], Silver et al. [35] demonstrated that negative regulators of the wingless pathway were targeted by *miR-315* and Kennell et al. [36] demonstrated that *miR-8* was able to regulate the wingless targets *distal-less* and *senseless*. Despite the biological significance of these results being limited in terms of wing imaginal disc development, as these miRNAs are not expressed in wing imaginal discs, the results are still important, since these signalling molecules have been implicated in the regulation of growth and proliferation and could therefore provide new insights into growth control and cancer. Indeed, the role of miRNAs in cancer is becoming more and more prominent [37, 38] and it is interesting to note that more than half of known human miRNAs are located near chromosomal breakpoints associated with cancer [39].

## Concluding remarks

The above examples give an insight into the large number of developmental functions carried out by miRNAs during *Drosophila* wing development. It seems likely that there are more functionally significant miRNA–mRNA interactions yet to be found in wing imaginal disc development, and certainly in the context of development and disease as a whole. There are still large number of predicted miRNA–mRNA interactions that still need to be validated, and it is also likely that there are many more biologically significant roles to be discovered for those miRNA-target pairs that have already been experimentally confirmed. Furthermore, the mechanisms by which miRNAs regulate their targets during development are becoming more complex. Thomsen et al. [40] demonstrate the existence of a 3′-UTR processing system that regulates target mRNA 'visibility' to their miRNAs, according to their developmental context and independently of the miRNAs themselves. Moreover, RNAs termed ceRNAs (competing endogenous RNAs), can compete with each other for binding to miRNAs to regulate each other's expression [41]. The potential for research in the miRNA field to lead to new treatments for disease is also huge and the discovery of the role of *bantam* in the regulation of growth and patterning during development is just one example of this. However, despite vast improvements in our understanding of how miRNAs function to regulate biological processes, there is still a long way to go until their full potential is utilized.

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