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MicroRNA Pathways in Flies and Worms: Growth, Death, Fat, Stress, and Timing

Minireview

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Drosophila **geneticists have uncovered roles for microRNAs in the coordination of cell proliferation and cell death during development, and in stress resistance and fat metabolism. In *C. elegans*, a homolog of the well-known fly developmental regulator *hunchback* acts downstream of the microRNAs *lin-4* and *let-7* in a pathway controlling developmental timing.**

MicroRNAs (miRNAs) are small RNA gene products that are believed to regulate the activity of messenger RNAs by antisense base pairing. The first genes recognized to encode microRNAs, *lin-4* and *let-7* of *C. elegans*, were identified on the basis of the developmental timing defects associated with loss-of-function mutations (Lee et al., 1993; Reinhart et al., 2000; reviewed by Pasquinelli and Ruvkun, 2002). It is now known that there are hundreds of microRNA genes in metazoans, many of which are evolutionarily conserved (reviewed by Carrington and Ambros, 2003).

Functions of Fly microRNA Genes

Until recently, no function had been assigned to any microRNA gene other than *lin-4* and *let-7* of *C. elegans*. That situation has changed dramatically, with the recent publication of papers from the laboratories of Steve Cohen (Brennecke et al., 2003) and Bruce Hay (Xu et al., 2003). Using genetic screens for morphologically abnormal flies, the Cohen lab had previously identified mutations affecting the *bantam* gene (Hipfner et al., 2002). *bantam* was then cloned based on its mutant phenotype, and found to produce a microRNA gene product that regulates cell proliferation and cell death (Brennecke et al., 2003; Figure 1). By similar genetic screens, in this case for mutations that alter *reaper*-induced apoptosis in the fly eye, the Hay lab identified mutations in *mir-14* that not only influence the expression of the cell death pathway, but also affect fat metabolism (Xu et al., 2003; Figure 1).

Animal microRNAs seem to recognize target messenger RNAs by imprecise base pairing, and in general probably elicit translational repression of their targets, as has been shown for *lin-4* of *C. elegans* (Olsen and Ambros, 1999). Brennecke et al. (2003) used computational methods to search for candidate *bantam* target genes, and identified the cell death inducer *hid* on the basis of finding several elements in the *hid* 3′ UTR that are partially complementary to *bantam* miRNA. Brennecke et al. (2003) further showed that the *hid* 3′ UTR is necessary and sufficient for repression of a transgene in flies, and that endogenous Hid protein is downregulated in response to the presence of *bantam* activity in vivo. *bantam* also mediates the developmental control of cell proliferation, probably in response to spatially graded signals such as *wingless*.

Does *mir-14* work like *bantam*? Yes and no. *bantam* and *mir-14* can both inhibit apoptosis induced by overexpression of cell death activators in the fly eye. However, unlike *bantam*, *mir-14* does not appear to promote cell proliferation. Also, *hid* is not predicted to be an antisense target of *mir-14*. Rather, Xu et al. (2003) found evidence that *mir-14* may, directly or indirectly, repress the cell death inducer *Drice* (Figure 1). Consistent with this model, the *Drice* transcript is predicted to contain *mir-14* complementary sequences, although there is not yet experimental confirmation that *Drice* is a direct target of *mir-14*. Remarkably, *mir-14* loss-of-function flies display increased sensitivity to a variety of stressful conditions, and also accumulate large quantities of lipid droplets in adipocytes. Xu et al. (2003) suggest that *mir-14* may normally function to regulate fat metabolism, apoptosis, and other physiological responses related to organismal stress (Figure 1).

These new *Drosophila* findings are exciting for a number of reasons. First, the involvement of *mir-14* and *bantam* in cell death in flies definitively shows that microRNAs play important regulatory roles outside of *C. elegans* and in arenas other than developmental timing. *bantam* also promotes cell proliferation, and therefore is formally an oncogene. Other miRNAs could also be important regulators of cell proliferation, even in vertebrates; human *mir-15* and *mir-16* are located in a chromosomal region commonly deleted in chronic lymphocytic leukemia, suggesting that these miRNAs may be tumor suppressors (Calin et al., 2002). Since *bantam* inhibits apoptosis, which is often associated with increased cell proliferation, *bantam* could function to coordinate these major components of tissue growth and homeostasis. By analogy, *mir-14* could function to coordinate multiple cellular responses to stress (Xu et al., 2003). Clearly, exciting questions remain to be addressed. Precisely how do *mir-14* and *bantam* engage with the cell death pathway, and what is the functional relationship between these two cell death regulators? What are the cell cycle regulators that *bantam* targets to promote proliferation? Finally, what upstream developmental and physiological pathways regulate the activities of *mir-14* and *bantam* microRNAs?

Another exciting aspect of these new functional studies of *Drosophila* microRNAs is the wonderful repertoire of sophisticated fly genetic tools now being applied to microRNA genes. Xu et al. (2003) employed the tried and true genetic screens for enhancers or suppressors of fly eye phenotypes, and identified *mir-14* loss-of-function alleles. Hipfner et al. (2002) found the *bantam* locus by screening for tissue overgrowth phenotypes caused by insertion of a modified P element (EP element). EP elements cause inducible overexpression of loci adjacent to the insertion site (Rorth, 1996), and hence can produce gain-of-function phenotypes. Although *bantam* deletions turned out to display a loss-of-function phenotype, the EP element gain-of-function

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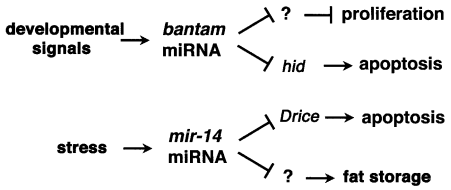

Figure 1. Roles for *Drosophila* miRNAs *bantam* and *mir-14*

bantam promotes cell proliferation in response to developmental signals and inhibits apoptosis (Brennecke et al., 2003). *mir-14* represses apoptosis and the accumulation of lipid droplets in adipocytes, perhaps as part of a stress response pathway (Xu et al., 2003). The cell death activators *hid* and *Drice* are potential posttranscriptional targets of *bantam* and *mir-14*, respectively. Hypothetical targets ("?") for the control of proliferation and fat storage are yet to be identified.

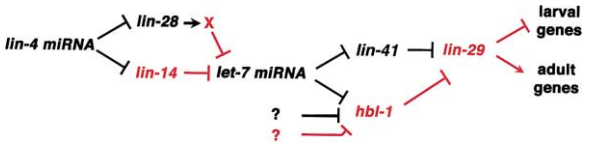

Figure 3. A Regulatory Cascade of *C. elegans* Heterochronic Genes

Based on epistasis and expression studies in Abrahante et al. (2003) and Lin et al. (2003); reviewed by Pasquinelli and Ruvkun (2002). Probable transcription factors are shown in red; all other regulators act posttranscriptionally. X represents a hypothetical gene proposed here to couple the LIN-28 cytoplasmic RNA binding protein to *let-7* transcription. The temporal regulation of *hbl-1* (*hunchback*) expression is complex, involving both transcriptional (red "?") and posttranscriptional (black "?") repression.

strategy may be particularly effective for identifying microRNA genes, especially those that act redundantly with other genes, and hence would be missed by loss-of-function screens. Another factor arguing in favor of gain-of-function phenotypic screens for microRNA genes is the relative insensitivity of small RNA genes to mutation, compared to much larger protein-coding genes.

The Brennecke et al. (2003) paper further describes a potentially powerful and general method for monitoring the anatomical pattern of expression of a given microRNA in vivo. A "sensor" transgene was employed that takes advantage of the potential of animal microRNAs to act as siRNAs. Whereas plant microRNAs naturally recognize target mRNAs by precise base pairing and thereby elicit target degradation through RNAi (Tang et al., 2003), animal microRNAs all seem to have only imprecisely matched targets. Nevertheless, an endogenous animal miRNA can elicit target degradation if presented with an experimentally introduced messenger RNA designed with precise complementarity to the miRNA (Hutvagner and Zamore, 2002; Zeng et al., 2002). Brennecke et al. (2003) harnessed this latent RNAi potential of *bantam* to detect the location of *bantam* expression in the fly (Figure 2). By this assay, *bantam* levels were shown to correlate with those areas of the wing imaginal disk that are most active in cell proliferation,

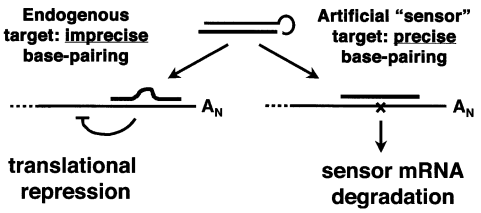

Figure 2. Two Modes of Gene Silencing by microRNAs

Ordinarily, animal microRNAs recognize targets by imprecise complementarity, and so they do not trigger target degradation by RNAi. Brennecke et al. (2003) employed a GFP reporter transgene as a microRNA sensor, with regions of the GFP mRNA engineered to be precisely complementary to the *bantam* microRNA. Thus, cells that express endogenous *bantam* miRNA are identified by a reduced GFP expression in those cells owing to microRNA-induced degradation of the complementary GFP messenger RNA.

consistent with a role for *bantam* in the promotion of cell proliferation.

hunchback and Developmental Timing

While the Brennecke et al. (2003) and Xu et al. (2003) papers show for the first time that miRNA genes function in flies, and in processes other than developmental timing, two other recent papers, one from Ann Rougvie's lab (Abrahante et al., 2003) and one from Frank Slack's lab (Lin et al., 2003), report evidence for conservation of developmental timing mechanisms between worms and flies. Specifically, a worm homolog of *hunchback*, a transcription factor known to control spatial patterning of the *Drosophila* embryo, was shown to function in the *C. elegans* developmental timing pathway, and to be regulated by a microRNA.

Both Abrahante et al. (2003) and Lin et al. (2003) describe screens for mutants with abnormal timing of hypodermal cell development. These screens yielded loss-of-function alleles of *hbl-1*, the closest worm homolog to the *Drosophila* segmentation gene, *hunchback* (Fay et al., 1999). *hunchback* is a zinc finger transcription factor well known for its role in establishing anterior-posterior differences in the *Drosophila* embryo. *hunchback* also functions during development of the fly central nervous system (CNS), where it regulates steps in a temporal sequence of cell fate choices (Isshiki et al., 2001). *hbl-1* in *C. elegans* seems to primarily affect developmental timing, suggesting similar developmental timing roles for *hunchback* in the worm hypodermis and in the fly CNS, and further suggesting that fly *hunchback* might also be regulated by miRNAs.

The discovery of a developmental timing role for *C. elegans hunchback* fills a gap in our understanding of the regulatory pathway of worm heterochronic genes. The heterochronic genes act during *C. elegans* larval development to distinguish one stage from another so that cells express stage-specific developmental programs (reviewed by Pasquinelli and Ruvkun, 2002). Mutants lacking the microRNA *let-7* fail to properly execute a larval-to-adult switch in hypodermal cell development. This switch requires LIN-29, an adult-specific zinc-finger transcription factor (Figure 3). It was known previously that *let-7* activates *lin-29* indirectly, by repressing the translation of *lin-41* messenger RNA (Slack et al., 2000). LIN-41 is a probable RNA binding protein, and so the *let-7–lin-41–lin-29* circuit seems to represent a cascade of posttranscriptional gene regulation. However, *let-7*

and *lin-41* alone cannot entirely account for the observed developmental regulation of *lin-29* gene activity; the *let-7* and *lin-29* null phenotypes are not equivalent, and *lin-41* mutations are only partially epistatic to *let-7* (Abrahante et al., 2003). What other temporal regulators collaborate with *let-7* and *lin-41* activities to specify the activation of *lin-29* at the end of the fourth larval stage?

Worms doubly deficient for both *lin-41* and *hbl-1* have much stronger developmental timing defects than either singly defective animal, suggesting that *lin-41* and *hbl-1* act in parallel pathways (Abrahante et al. 2003, Lin et al., 2003). Both the *lin-41* and *hbl-1* 3′ UTRs contain partial complementarity to *let-7* RNA, suggesting that *let-7* could act by repressing both *lin-41* and *hbl-1* (Figure 3). Moreover, the *lin-41;hbl-1* double loss-of-function is strongly epistatic to *let-7* loss-of-function, consistent with the model that *let-7* controls the timing of adult cell fates by repressing *lin-41* and *hbl-1* in parallel (Figure 3).

C. elegans hunchback levels decrease during development, as a consequence of what appears to be tissue-specific transcriptional and posttranscriptional regulation. In the nervous system, *hbl-1* seems to be translationally repressed by *let-7* microRNA, acting via the *hbl-1* 3′ UTR (Abrahante et al., 2003; Lin et al., 2003). In the nondividing lateral hypodermal (skin) cells, *hbl-1* is also translationally repressed, but independently of *let-7*, implicating perhaps one or more other microRNAs. Finally, there seems to be a UTR-independent, early transcriptional component to *hbl-1* downregulation in the dividing population of hypodermal cells. Further work is required to identify the pathways and mechanisms responsible for the *let-7*-independent regulation of *hbl-1* (Figure 3).

How conserved are the fly and worm *hunchback*-related pathways? Fly *hunchback* is subject to spatially restricted translational repression in early embryos (Wharton and Struhl, 1991). Could miRNAs serve as specificity factors in the translational regulation of *hunchback* messenger RNA during fly embryogenesis, analogous to the role of *let-7* in the repression of *hbl-1* in the worm nervous system? Does *hunchback* regulate progression of cell fates in *Drosophila* CNS, and in the *C. elegans* hypodermis, by a conserved pathway? There are hints that this could be so: *let-7* is conserved in *Drosophila* (Pasquinelli et al., 2000) and the fly hunchback 3′ UTR contains potential *let-7* complementary sites (Abrahante et al., 2003; Lin et al., 2003). Further, *Drosophila hunchback* regulates the expression of *kruppel,* a zinc-finger protein of the LIN-29 class. Perhaps a common ancestor of worms and flies contained a *let-7* microRNA that temporally regulated *hunchback* and LIN-41, thereby restricting the timing of *kruppel*/LIN-29 expression.

Conclusions

Why should microRNA-based gene regulatory mechanisms be conserved? In particular, why would animals conserve the widespread use of translational repression by miRNAs through imprecise target recognition? Assuming that *let-7*, *bantam*, and *mir-14*, like *lin-4*, repress translation of their targets by intervening at a step after translational initiation (Olsen and Ambros, 1999), what would be the utility of this particular mechanism, in comparison to the evident potential of animal microRNAs to trigger degradation of a precisely matched mRNA target (Hutvagner and Zamore, 2002; Zeng et al., 2002; Brennecke et al., 2003)? Clues to understanding these questions may come from the observation that miRNAs seem to be dosage-sensitive regulators of downstream biological pathways. The Xu et al. (2003) and Brennecke et al. (2003) papers report that the frequency of cell death in the *Drosophila* eye can be exquisitely sensitive to the dosage of *mir-14* or *bantam* microRNA, respectively. Dosage sensitivity is also a hallmark of the regulation of adult fates by *let-7* in the worm heterochronic gene pathway (Slack et al., 2000). This dosage sensitivity suggests that miRNAs could exercise a finely tuned and perhaps very sensitive control of target gene expression. By regulating gene expression at the ultimate output of genetic information flow, the ribosome::mRNA complex, a microRNA can exert an effect on target gene expression that is potentially immediate, rapidly reversible, and very responsive to developmental or physiological signals that influence miRNA activity. This mode of gene regulation would also be well suited for the concerted repression of multiple genes, independently of variables such as mRNA turnover rates and transcriptional mechanisms.

The degree of evolutionary conservation and functional analogy among pathways involving microRNAs remains to be uncovered through further studies of the four microRNAs that have been assigned functions by genetics (*lin-4* and *let-7* of *C. elegans*, *mir-14* and *bantam* of *Drosophila*), and through genetic analysis of other miRNA genes. However, these new findings already tell us that microRNAs are deployed in a broad range of developmental and physiological contexts, and that there may be more overlap than previously suspected between developmental timing and spatial patterning pathways in worms and flies, and perhaps among animals in general.

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