Published in final edited form as:

Curr Top Dev Biol. 2012 ; 99: 201–235. doi:10.1016/B978-0-12-387038-4.00008-2.

Exploiting *Drosophila* genetics to understand microRNA function and regulation

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Abstract

Although a great deal is known about the identity, biogenesis, and targeting capacity of microRNAs (miRNAs) in animal cells, far less is known about their functional requirements at the organismal level. Much remains to be understood about the necessity of miRNAs for overt phenotypes, the identity of critical miRNA targets, and the control of miRNA transcription. In this review, we provide an overview of genetic strategies to study miRNAs in the *Drosophila* system, including loss- and gain-of-function techniques, genetic interaction strategies, and transgenic reporters of miRNA expression and activity. As we illustrate the usage of these techniques in intact *Drosophila*, we see certain recurrent themes for miRNA functions, including energy homeostasis, apoptosis suppression, growth control, and regulation of core cell signaling pathways. Overall, we hope that this exposition of *Drosophila* genetic techniques, well-known to the legions of fly geneticists and used to study all genes, can inform the general miRNA community that focuses on other biochemical, molecular, computational, and structural avenues. Clearly, it is the combination of these myriad techniques that has accelerated miRNA research to its extraordinary pace.

1. Introduction

microRNAs (miRNAs) are short regulatory RNAs that mediate broad networks of post-transcriptional repression, with consequences for diverse aspects of development and physiology (Flynt and Lai, 2008). Correspondingly, there is growing appreciation of how human disease and cancer is driven by aberrant or dysfunctional miRNA activity. The majority of miRNAs are generated by a characteristic biogenesis pathway (Kim et al., 2009). In animal cells, this consists of stepwise processing of hairpin precursor transcripts by the Drosha and Dicer RNase III enzymes to yield a ~22 nucleotide (nt) small RNA duplex, of which one strand is preferentially loaded into an Argonaute protein and guides it to regulatory targets. In addition, several alternate pathways generate miRNAs via Drosha-independent or Dicer-independent pathways (Yang and Lai, 2011).

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As with other classes of Argonaute-mediated small RNAs, i.e. siRNAs and piRNAs, the miRNA serves as sequence-specific guide that recruits the Argonaute complex to target transcripts (Czech and Hannon, 2010). In animal cells, the key information for miRNA target identification lies within the 5' end of the small RNA (the "seed"), since ~7 nt complements to positions 2-8 of the miRNA are capable of mediating substantial repression (Brennecke et al., 2005; Doench and Sharp, 2004; Lai, 2002). Consequently, miRNA targets can be predicted genome-wide via conserved miRNA seed matches (Bartel, 2009); location within the transcript, local sequence bias and secondary structure, and other RNA binding proteins can also influence target efficacy. The abundance of sequenced and aligned genomes alignments provides evidence that a large fraction of well-studied metazoan transcripts bear conserved miRNA target sites (Friedman et al., 2009; Jan et al., 2010; Mangone et al., 2010; Ruby et al., 2007b). The endogenous impact of miRNA-mediated repression should be larger still, in light of the facts that many functional sites are not conserved, and that at least certain types of non-seed sites can confer repression (Brodersen and Voinnet, 2009).

The wealth of information from comparative genomics, as well as transcriptome- and proteome-based analyses (Baek et al., 2008; Guo et al., 2010; Lim et al., 2005), provides ever-increasing information on the scope of miRNA-mediated repression. Nevertheless, such studies have not provided a straightforward route towards predicting the phenotypic consequences of altering miRNA activity in the context of the whole organism (Smibert and Lai, 2008). Ironically, some of the best-understood biological usages of miRNAs derived from studies conducted prior to the formal recognition of miRNAs. In particular, *C. elegans* genetics permitted the first (lin-4) and second (let-7) identified miRNAs to be placed within regulatory hierarchies that control developmental timing, and identified their key direct target genes (Lee et al., 1993; Moss et al., 1997; Reinhart et al., 2000; Wightman et al., 1993). In addition, genetic studies of the *Drosophila* Notch pathway identified key miRNA target genes prior to the cloning of miRNAs (Lai et al., 1998; Lai and Posakony, 1997; Lai and Posakony, 1998), and led to the concept of 7 nt complements to miRNA 5' ends as animal miRNA binding sites (Lai, 2002). Therefore, genetic analysis was central to revealing the existence and mechanism of miRNAs.

The genome and transcriptome of *Drosophila melanogaster* has been extensively scoured for miRNA genes, and its current state of annotation is perhaps the deepest amongst any animal species (Berezikov et al., 2011; Chung et al., 2011), and includes loci derived from several non-canonical pathways (Flynt et al., 2010; Okamura et al., 2007; Ruby et al., 2007a). Deletion mutations of over thirty well-conserved *Drosophila* miRNA genes, comprising eighteen genomic loci/clusters, have been described, and collectively reveal critical biological requirements for miRNAs. Many other miRNA loci have been associated with compelling gain-of-function phenotypes, and still others are "interesting" from the point of view of cell- or tissue-specific expression patterns (Aboobaker et al., 2005; Ruby et al., 2007b), conserved targeting of well-studied protein-coding genes (Ruby et al., 2007b; Stark et al., 2007), or principles that relate cohorts of miRNA target genes (Lai, 2002; Stark et al., 2005). Altogether, studies in the *Drosophila* model have richly illuminated our understanding of miRNA-mediated regulation (Smibert and Lai, 2010).

In this review, we concentrate on what has been learned about the biological usage of miRNAs from fly genetics. The *Drosophila* system is privileged to have had many researchers devoted to technology development over the decades, resulting in a battery of powerful methods for genetic analysis. We do not intend to present an exhaustive compilation of fly techniques; rather, we summarize the genetic toolbox that has been most utilized for miRNA research. We hope that these detailed illustrations of how specific techniques have illuminated *Drosophila* miRNA biology may provide a useful reference and comparison for those studying other animal models (e.g. *C. elegans*, zebrafish, mouse, and cultured mammalian cells). As well, in the course of discussing landmark and recent *Drosophila* miRNA literature, we specifically point out general implications from fly genetics that have particular bearing on the collective endeavor of studying miRNA functions.

2. Generation of miRNA mutants

The gold standard for studying gene function in model organisms is by mutant/deletion alleles within the intact organism. In *Drosophila* and *C. elegans*, large-scale genetic screens using chemical mutagens or transposons were instrumental in elucidating genes and pathways that control development. The small size of miRNAs makes them poor mutagenesis targets. Only through extremely deep genetic screens, and heroic and stubborn positional cloning efforts, have rare point mutations in miRNA genes been recognized in nematodes (Figure 1A) (Chalfie et al., 1981; Johnston and Hobert, 2003; Lee et al., 1993; Reinhart et al., 2000). Similarly, a handful of *Drosophila* miRNA loci were associated with notable loss-of-function phenotypes prior to realization of their encoded small RNAs (Brennecke et al., 2003; Cayirlioglu et al., 2008; Hardiman et al., 2002; Hipfner et al., 2002; Raisin et al., 2003; Xu et al., 2003). However, in flies as in all other species, by far the vast majority of miRNA mutants have been studied by reverse genetics. We begin by reviewing the methods by which *Drosophila* miRNA mutant alleles have been generated.

2.1. Transposon-induced miRNA mutants from forward genetic screening

The “taming” of transposable elements for experimental use has been invaluable for the creation of mutant alleles, provide entry points to clone genes, probe expression patterns, and of course facilitate a majority of *Drosophila* genetic experiments. The general utility of transposon insertion collections for forward phenotypic screening, as well as serving as an allele repository, has resulted in mapping and curation of several hundred thousand transposon mobilization events over the years. The 20 year-old *Drosophila* Gene Disruption Project (GDP) is a prominent community resource project that seeks to isolate insertions in all *Drosophila* genes. In addition to an immense work from the core project members (Bellen et al., 2011), the GDP has also consolidated transposon collections from many other laboratories and even companies (e.g. the Exelixis and GenExel collections) (Bellen et al., 2004; Deak et al., 1997; Lee et al., 2005; Spradling et al., 1999; Thibault et al., 2004).

Some transposons, most notably the *P* element, prefer to insert in active promoters (Figure 1B). When viewed alongside genome annotation tracks (e.g. <http://flybase.org/>), this preference becomes quite obvious, with many insertions piling up at the 5' ends of annotated genes. Of course, not every insertion represents a promoter, and many insertions are not

associated with annotated genes. However, in the case of hotspots associated with multiple insertions, one can infer that some type of promoter has been tagged. A number of these “orphan” insertion hotspots were later recognized to identify alternative 5' exons of protein-coding genes (Manak et al., 2006), but a number of them proved to identify miRNA genes (Brennecke et al., 2003; Cayirlioglu et al., 2008; Hipfner et al., 2002; Raisin et al., 2003).

Transposons frequently affect the expression of the inserted loci, thereby acting as mutant alleles (Figure 1B). One of the very first *Drosophila* miRNAs reported was recovered on the basis of a *P* insertion in the *mir-14* locus, which is indeed a *P* hotspot (Xu et al., 2003). In this study, flies were sensitized by expression of the pro-apoptotic gene reaper in the developing eye (*GMR>reaper*), which induces small, rough eyes. This stock was crossed to a collection of lethal *P* insertions to find ones that could modify the rough eye phenotype, thus representing loci that putatively affect cell death. An insertion in *mir-14* enhanced *GMR>reaper*, yielding smaller eyes and increased pupal lethality. This proved to be due to loss of *mir-14*, since the enhancement of *GMR>reaper* was reverted by precise excision of the *P* insertion, recapitulated in a specific deletion of the *mir-14* locus, and rescued by reintroduction of *mir-14* genomic DNA. In fact, overexpression of miR-14 potently suppressed *GMR>reaper*, rescuing eyes almost back to wildtype. This was not specific to reaper, since ectopic miR-14 also strongly rescued the small rough eyes induced by other pro-apoptotic transgenes such as *GMR-hid* and *GMR-grim* (Xu et al., 2003). These genetic tests established an intimate connection between miR-14 and suppression of pro-apoptotic factors.

Another *Drosophila* locus identified on the basis of forward loss-of-function screening of *P* insertions was *mir-279* (Cayirlioglu et al., 2008). This mutant was identified by clonal screening for mutations that affected CO₂-sensing neurons in the olfactory system. These neurons normally arise only in the antenna, but a *P* element insertion near *mir-279* caused ectopic CO₂-sensing to be specified in the maxillary palp. As with *mir-14*, the *mir-279* insertion acted as a loss-of-function mutant, since these phenotypes were recapitulated by independent deletions of the *mir-279* locus. Therefore, miR-279 suppresses target genes in the maxillary palp to prevent the specification of CO₂-sensitive neurons. One of its more critical direct targets is the zinc finger transcription factor encoded by *nerfin-1*, since heterozygosity for this gene substantially decreased the ectopic CO₂-sensing neurons in *mir-279* mutant palps (Cayirlioglu et al., 2008). This implied that de-repression of nerfin-1 was critical for generating these neurons; nevertheless, there must be other relevant miR-279 targets since misexpression of nerfin-1 was not sufficient to generate CO₂-sensing neurons in palps.

These examples highlight how the context of forward genetics provides insight regarding the *in vivo* function of miRNAs. Even to this day, despite great improvements in our understanding of parameters underlying miRNA target regulation and comprehensive set of sequenced *Drosophilid* genomes (Bartel, 2009; Stark et al., 2007), and substantial additional study of miR-14 genetics (Varghese and Cohen, 2007; Varghese et al., 2010), the direct connection of miR-14 to the apoptosis pathway remains unclear. Nevertheless, the dose sensitivity of *mir-14* for reaper-mediated cell death, as well as the potent capacity of miR-14 to suppress the activity of multiple other pro-apoptotic triggers, supports an intimate

connection of miR-14 and cell death. This remains to be deciphered in the future. Likewise, *mir-279* was known for some time to be expressed specifically in the nervous system from embryo studies (Aboobaker et al., 2005), but a specific and essential functional connection to a specialized population of CO₂-sensing neurons in the brain certainly could not have been anticipated from target predictions. Therefore, as was so elegantly illustrated by studies of *C. elegans* miRNAs to emerge from forward genetics—*lin-4* and *let-7* that control heterochronic timing (Lee et al., 1993; Reinhart et al., 2000) and *lsy-6* which controls left-right asymmetry of ASE neurons (Johnston and Hobert, 2003), phenotype-based genetic studies can lay an invaluable foundation for the biological study of miRNAs.

2.2. Deletion of miRNA loci by imprecise excision of *P* elements

Unfortunately, rather few loss-of-function mutants of miRNA loci have had the privilege of emerging from forward genetic studies. Consequently, studies of miRNA biology have been dominated by reverse genetic approaches. Although transposon movement is generally mediated by specific recombination events, a useful feature of *P* elements is their tendency to excise imprecisely at low frequency, thereby inducing local deletions (Figure 1C). Therefore, even if the starting insertion does not disrupt expression, as with the original insertions in *mir-14* and *mir-279*, one can utilize imprecise *P* excision to delete neighboring DNA yielding null alleles. This is commonly used to delete protein-coding genes, and has similarly been applied to miRNA genes where possible. Since many miRNA genes have been tagged by *P* elements, this has permitted them to be deleted by imprecise excisions, including *bantam* (Hipfner et al., 2002), *mir-7* (Li and Carthew, 2005), *mir-8* (Karres et al., 2007), *mir-14* (Xu et al., 2003), *mir-279* (Cayirlioglu et al., 2008), *mir-310/311/312/313* cluster (Tsurudome et al., 2010), and *mir-263a* (Hardiman et al., 2002); a deletion of *mir-iab-4/8* was generated by *P* element-induced gene conversion (Bender, 2008). Moreover, perusal of *P* insertions curated by FlyBase (<http://flybase.org/>) reveals a number of other miRNA insertion hotspots (e.g., *mir-275/305*, *mir-276a*, *mir-282*, etc.) for which deletions could presumably be generated in a straightforward manner.

Studies of *mir-263a* illustrate a particularly interesting case of how reverse genetics uncovered a key developmental role of a miRNA (Hilgers et al., 2010). Over twenty years ago, the *P* element *lacZ* enhancer trap, *E8-2-46*, was isolated on the basis of its sensory organ-specific expression (Bier et al., 1989). Molecular characterization of the region identified several long transcripts lacking substantial open reading frames (Hardiman et al., 2002). Nevertheless, generation of deletion alleles by *P* excision revealed that this locus, *bereft*, was important for at least one class of sensory organs, the interommatidial bristles (IOBs) of the eye. Subsequent computational discovery of miRNAs elucidated the related, but unlinked, loci *mir-263a/mir-263b* (Lai et al., 2003). The former was located just downstream of *bereft*, raising the possibility of a relation between these loci.

This indeed proved to be the case. Although the original *bereft* deletions did not remove the *mir-263a* hairpin itself, Northern analysis showed they lacked mature miR-263a just the same (Hilgers et al., 2010). Moreover, specific mutants of *mir-263a* generated by homologous recombination (a technique discussed in Section 2.5), failed to complement the original *bereft* alleles and recapitulated *bereft* phenotypes in IOB organs. Further analysis of

pupal development revealed that *bereft* mutants specify the sensory organ precursors for IOBs, and also execute this cell lineage normally. However, the shaft cells of IOB organs succumb to apoptosis, indicating that miR-263a is anti-apoptotic. This interpretation is supported by the fact that loss of *mir-263a* can be compensated by misexpression of the anti-apoptotic viral protein p35.

The pro-apoptotic gene *hid* was found to be a key target gene of miR-263a/b, since heterozygosity for *hid* could substantially rescue *mir-263* mutants, and miR-263 could suppress luciferase-*hid* 3'UTR sensors (Hilgers et al., 2010). Curiously, miR-263a and miR-263b are rather unusual family members in that their seed regions have diverged (at position 2). According to current knowledge, continuous Watson-Crick seed pairing is the major determinant for animal miRNA target recognition, so that seed changes are expected to redirect targeting capacity (Bartel, 2009). The relevant miR-236a/b target sites in the *hid* 3'UTR are atypical in that none of the sites are canonical 2-8 matches. This might be compensated by the existence of multiple target sites; for example, miR-263a pairs to 4 sites in *hid* exhibiting 3-9 pairing (and 1G:U), 1-7 pairing, or 2-7 pairing (2 sites).

Two aspects of the miR-263 story are particularly instructive with respect to general miRNA studies. First, it shows that a miRNA locus was actually deleted and shown to have a compelling developmental defect in the “pre-miRNA era”, but was not initially deciphered due to lack of knowledge of the associated small RNA. One may wonder whether other miRNA loci were studied genetically over the years, but the associated small RNA was not appreciated. Second, it highlights how genetics led to elucidation of a miRNA:target relationship that is critical for normal animal development, but that could not have been found by standard miRNA target predictions, owing to its non-canonical sites. Thus, one should be circumspect in utilizing genome-wide target predictions, which comprise powerful information, but may not include all critical targeting relationships.

2.3. Other transposons

Owing to the insertion site preference of *P* elements, the yield of new genomic insertions has steadily decreased over the years. The use of other transposons with distinct insertion mechanisms can broaden the distribution and coverage of these collections. One widely used element is piggyBac, which exhibits broader insertion range across the genome relative to *P*, including the capacity to insert within genes and introns (Figure 1B) (Bellen et al., 2004). Because of this, piggyBac has broader possibilities to disrupt gene expression or function. However, a disadvantage of piggyBac is that it essentially only excises precisely. This disadvantage can be ameliorated by adding other functionalities to the vector backbone. For example, one can place recombinogenic sequences such as FRT onto the transposon. When placed *in trans* with another FRT-bearing insertion, and in the presence of FLP recombinase, one can induce deletions of genomic DNA between the FRT sites (Figure 1D). The possibility to generate molecularly defined deletions has motivated the creation of extensive FRT-piggyBac collections for custom deletions (Parks et al., 2004; Ryder et al., 2007; Thibault et al., 2004). Several *Drosophila* miRNAs, including *mir-1* (Kwon et al., 2005), *mir-184* (Iovino et al., 2009) and *mir-284* (Karr et al., 2009), were deleted in this fashion. In the future, “finishing” of the *Drosophila* Gene Disruption Project to 95% of all genes will be

facilitated by yet other transposons. In particular, Minos combines a broad capacity for random insertion throughout the genome with the possibility for imprecise excisions (Figure 1B) (Bellen et al., 2011).

2.4. miRNA alleles induced by homologous recombination

Since the vast majority of miRNA loci lack useful transposon insertions, or reside in introns where deletions would affect both miRNA and protein coding genes, targeted methods must be used to generate alleles (Figure 1E). Of course, homologous recombination (HR) has been widely used in unicellular organisms and mice for quite some time, but a strategy to perform this in *Drosophila* was only developed a decade ago (Rong and Golic, 2000; Rong and Golic, 2001; Rong et al., 2002). The main limitation in fly, compared to mouse, is the inability to re-generate an intact organism from an ES-like cell that can be propagated and manipulated in culture. HR requires the introduction of linear molecules, which unlike circular or supercoiled forms, are recombinogenic. This was finally solved by a two-step procedure for generating the HR substrate *in vivo*, in the germline, by using an integrated vector with flanking FRT sites and an internal rare restriction endonuclease site (I-SceI). Expression of FLP recombinase in the germline excises the targeting vector, converting it into a circular form that is then linearized by I-SceI cleavage. HR is still a relatively rare event, requiring extensive screening to identify targeted alleles. Nevertheless, it has proven to be a reasonably reliable technique that has been adapted to generate many designer alleles, including a number of miRNA deletions.

A growing number of *Drosophila* miRNA mutants have been made by HR, including *mir-1* (Sokol and Ambros, 2005), *mir-309/3/286/4/5/6-1,2,3* cluster (Bushati et al., 2008), *mir-278* (Teleman et al., 2006), *let-7/mir-125/mir-100* cluster (Caygill and Johnston, 2008; Sokol et al., 2008), *mir-9a* (Li et al., 2006), *mir-12/283/304* cluster (Friggi-Grelín et al., 2008), *mir-31a* (Weng et al., 2009), *mir-263* and *mir-263b* (Hilgers et al., 2010). Their study has uncovered diverse aspects of development and physiology regulated by miRNAs, many of which will be discussed in subsequent sections. We highlight here one example from targeted knockout of *mir-9a*.

HR-induced deletions of *mir-9a* exhibit several mutant phenotypes that manifest in adult morphology (Li et al., 2006). First, these mutants develop some ectopic mechanosensory bristle organs on their back (“notum”). Although this effect is somewhat variable and mild, the specification of extra organs indicates that the full program of peripheral neurogenesis has been activated ectopically. More notably, *mir-9a* mutant animals exhibit fully penetrant loss of wing tissue, preferably along the posterior edge; this is a very easily noticeable phenotype. Two main target genes have been assigned to these phenotypes, the zinc finger transcription factor *senseless* and the LIM domain transcription cofactor *dLMO* (Bejarano et al., 2008; Biryukova et al., 2009; Li et al., 2006).

Interestingly, deregulation of both targets contributes to both phenotypes, although not equally. *senseless* and *dLMO* are both proneural genes, so it makes sense that their derepression can promote neurogenesis; the extent of ectopic bristles is mitigated in *mir-9a* mutant animals that are also heterozygous for either *senseless* or *dLMO* (Bejarano et al., 2008; Li et al., 2006). Both of these targets induce loss of wing tissue when misexpressed,

although the wing is especially sensitive to *dLMO*. High level misexpression of *Senseless* is required to induce loss of wing margin (Nolo et al., 2001), whereas mere duplication of the *dLMO* locus can induce loss of wing margin (Lifschytz and Green, 1979). Consequently, heterozygosity for *senseless* provides mild rescue to the *mir-9a* wing defect, but heterozygosity for *dLMO* provides a complete rescue (Bejarano et al., 2008; Biryukova et al., 2009; Li et al., 2006).

There are many substantial conclusions relevant to general miRNA biology to be drawn from studies of miR-9a. First, it is important to recognize that bristles and wings are two of the most intensely studied aspects of *Drosophila* development. Nevertheless, reverse genetics of *mir-9a* revealed its requirement in these two well-studied systems, indicating that it was somehow “missed” by 100 years of genetic screening. Second, it has been posited that many miRNA mutants may have subtle defects due to overlapping functions of related family members. This has proven the case with some miRNA families, although not for many others (Abbott et al., 2005; Alvarez-Saavedra and Horvitz, 2010). miR-9a has two nearly identical paralogs in *Drosophila*, miR-9b and miR-9c, yet the single *mir-9a* deletion exhibits compelling phenotypes. We do not know whether multiple *mir-9* mutants may exhibit stronger effects, yet it is clear that mutants in individual family members can have strong effects. In fact, the very first miRNAs recognized, lin-4 and let-7, are both members of miRNA families (Ambros et al., 2003), yet they clearly have potent non-redundant functions. Third, it is worth commenting that deciphering reverse genetics can depend strongly on the phenotypic richness of the system. The wing defect of *mir-9a* mutants is unmistakable; however, the few extra dorsocentral bristle organs are evident only because of the precision with which they are specified. One may have easily missed this phenotype, and one wonders whether an analogous vertebrate phenotype, say, having a few extra whiskers on the face of a mouse, might be noticeable. Finally, it is salient to mention that derepression of different targets of a miRNA may have distinct consequences in different locations of miRNA expression. We will return to this point later in the review (Section 4.3).

2.5. On the importance of rescuing mutant phenotypes

It cannot be over-emphasized that the careful practice of genetics involves a certain amount of legwork to prove that a given mutation is causal to an observed phenotype. Beyond the notes written on a stock label, there is always the possibility of additional unanticipated mutations that could underlie a phenotype of interest. For example, many *Drosophila* miRNA alleles were generated by transposon-mediated aberrations. *P* elements are known to incur a reasonably high frequency of hit-and-run events, such that the gene tagged by a transposon is not necessarily the same locus responsible for an associated phenotype. There may be other genes that were mutated before the transposon landed in its final spot, or transposon mobilization may have left damaged copies in the genome that are not easily detected by routine PCR checks. It is more effort to induce alleles by HR, but this technique allows aberrations to be generated much more precisely than by random transposon-induced deletions. Nevertheless, it is well-documented that the process of HR has a high frequency of inducing second-site mutations (O’Keefe et al., 2007). If one is unlucky, these might occur in some biased fashion leading to their presence on independent HR events. Worse yet, the homology arms themselves might affect neighboring genes in a systematic fashion.

Finally, the practice of keeping *Drosophila* stocks in a balanced state against chromosomes that suppress recombination permits unlinked mutations to accumulate over time.

Therefore, while one may be fortunate to be able to order miRNA loci tagged with *P* elements from public stock centers, or to have generated one's own miRNA alleles using HR, one should be cautious in relating observed phenotypes to the mutated locus. A few tests can be performed. In the case of transposon alleles, one can ask if a precise excision reverts the phenotype; if the phenotype persists, it may be a sign that it is due to some unlinked mutation. For example, in the original *mir-14 P* lethal stock identified as a suppressor of *GMR-reaper*, lethality was due to a background mutation and not by loss of *mir-14 per se* (Xu et al., 2003); of course, the apoptosis-modifying activity proved *mir-14*-dependent.

One may also seek to obtain independent alleles, and show that the phenotype is recapitulated in all of them, as was done with *mir-279* (Cayirlioglu et al., 2008). This is not a foolproof test, and in unlucky cases independent *Drosophila* stocks have been found to contain the same, unlinked mutation that was responsible for the observed phenotype (Roegiers et al., 2009). Generally speaking, though, this test provides a good measure of confidence. A powerful resource for "second alleles" is the *Drosophila* deficiency collection (Lindsley et al., 1972). This comprises a collection of balanced fly stocks that remove defined portions across >95% of the euchromatic genome. Therefore, it is a simple matter to place one's mutation of interest *in trans* to a deficiency of the region, which effectively acts as a null allele.

However, the most convincing test that an identified mutation is causal to phenotype is to rescue the mutant by reintroducing the gene product. One method is to re-express the miRNA in the mutant background; often this might be done using the Gal4-UAS system (see Section 3). However, as misexpression of miRNAs often induces dominant phenotypes, it can be difficult to achieve the "right" amount of expression *in vivo*. Another option is to re-introduce the genomic locus as a transgene, so that miRNA expression is subject to endogenous transcriptional control. Historically, this was challenged by the paucity of miRNA promoters and cis-regulatory elements known (see also Section 5), and the size limitation of inserts for efficient *P* element transgenesis (~15 kb). However, these issues have been obviated by the development of phiC31 transgenesis systems capable of integrating large inserts with high efficiency. Recently, collections of BAC (Venken et al., 2009) and fosmid (Ejsmont et al., 2009) inserts that tile across the *Drosophila* genome were made publicly available. This makes it convenient to simply order a genomic rescue for nearly every miRNA (and protein-coding) gene; the limitation being rare loci that cover in excess of >120 kb.

As many miRNA loci have relatively subtle phenotypes, and are implicated in maintaining organismal robustness (see Carthew review in this issue?), it becomes increasingly critical to determine that any observed difference of interest between a mutant and a wild-type reference is definitively due to loss of the miRNA, as opposed to strain background mutations. Therefore, one may wish to obtain such a rescuing genomic transgene early in the process of generating or analyzing miRNA mutants.

3. The Gal4-UAS system for transgene activation

The binary Gal4-UAS system comprises the backbone of a great deal of *Drosophila* research (Brand and Perrimon, 1993). This flexible, and simple, system permits researchers to conduct sophisticated genetics with little more base knowledge than the ability to sort females from males to set up crosses. The system comprises the transgene encoding the yeast transcription factor Gal4 as a “driver”, and a “responder” transgene linked to Gal4 binding sites (upstream activating sequence, or “UAS” sites). These components are kept in separate stocks, and Gal4 by itself does not usually induce obvious defects; only by crossing to bring the driver and responder transgenes into the same animal is the responder transgene expressed for phenotypic evaluation. There are many hundreds of fly stocks expressing Gal4 in various spatial and temporal patterns, facilitating diverse tissue-specific misexpression experiments. The Gal4 collections are complemented by many thousands of fly stocks containing UAS-responsive genes, either generated by directed cloning of individual genes or from mobilization of UAS-bearing transposons around the genome (often termed “EP” elements”). These resources permit a mind-boggling number of *in vivo* misexpression experiments to be conducted (Rørth et al., 1998), with little more knowledge required than to sort males from females to set up crosses.

3.1. Misexpression of miRNAs from genomic EP insertions

While the Gal4-UAS system was originally developed for the purpose of conducting gain-of-function analysis of protein-coding genes (Brand and Perrimon, 1993), it has found wide adoption for the misexpression of RNAi transgenes for knockdowns (Dietzl et al., 2007; Kennerdell and Carthew, 2000). Moreover, as mentioned above, a number of miRNA loci serve as *P* hotspots, and are thus represented in “EP” or other UAS-responsive insertion collections (Figure 2A). A number of these miRNA insertions routinely score as hits in diverse gain-of-function screens (including *mir-7*, *mir-8*, *mir-14*, *mir-282*, *mir-278*, *mir-310-313* cluster, and *bantam*), implying that this system is effective for misexpression of miRNAs. This is probably due to the endogenous transcription of most miRNAs by RNA Polymerase II (Lee et al., 2004), as with protein-coding genes.

Indeed, many of these miRNA hotspots emerged from gain-of-function screens conducted prior to the general recognition of miRNAs in *Drosophila* in 2001 (Lagos-Quintana et al., 2001), or prior to the initial large-scale annotations of *Drosophila* miRNAs in 2003 (Aravin et al., 2003; Lai et al., 2003), and thus were not likely recognized as miRNA loci at the time of their genetic isolation. Examples of miRNA hits in “pre-miRNA era” screens include that a gain-of-function screen for loci that could affect adult bristle sensory organs recovered *mir-7*, *mir-278* and *bantam* (Abdelilah-Seyfried et al., 2000), a misexpression screen for genes affecting motor axons and synaptogenesis identified *bantam* (Kraut et al., 2001), and that an ectopic expression study for modifiers of dorsal thorax formation hit *mir-278* (Pena-Rangel et al., 2002). Now that miRNAs are better annotated in the *Drosophila* genome, nearly all genome-wide misexpression studies end up recovering one or more of the set of EP-miRNA hotspots.

Most of these miRNA misexpression hits have yet to be followed up, but they provide possible entry points to understanding miRNA biology. In addition, EP-induced dominant

phenotypes provide a genetic entry point to generate specific miRNA mutants. Nearly all miRNA alleles are straight deletions, but having a range of point mutations could provide interesting insights, as with protein-coding genes. In the case of *mir-278*, its misexpression generates tissue overgrowths (Nairz et al., 2006), a property that was used in a reversion screen. *EP-mir-278* flies were mutagenized and screened for loss of the ability to induce large eyes. A single point mutant in the seed region of miR-278 was isolated as a revertant from ~10,000 animals (Nairz et al., 2006), a substantial but by no means daunting number for *Drosophila* genetics (Figure 1A). One could imagine that additional reversion screening of this and other EP-miRNA loci could produce insights regarding the necessity of 3' regions of miRNAs, for example.

3.2. Directed analysis of UAS-miRNA transgenes

The realization that miRNAs can be ectopically expressed effectively using the Gal4-UAS system has led to the production of many UAS-miRNA transgenes, and extensive illustrations of the detrimental consequences of ectopic miRNAs (Figure 2A). One notable set of phenotypes from miRNA gain-of-function emerged from studies of the *Drosophila* Bithorax-Complex (BX-C) locus *mir-iab-4/mir-iab-8*, which generates miRNAs from bidirectional transcription and processing of the same genomic hairpin locus (Bender, 2008; Stark et al., 2008; Tyler et al., 2008). Amongst the targets of these miRNAs are several homeobox genes in the BX-C, which specify the identities of various abdominal segments (Lewis, 1978). One such gene is *Ultrabithorax* (*Ubx*), which normally represses the wing development program in the segment that generates the haltere; thus *Ubx* mutants bear homeotic transformation of halteres into an extra pair of wings. Misexpression of *mir-iab-4* can directly represses *Ubx* in developing halteres, causing them to transform partially into wings (Ronshaugen et al., 2005); while many miRNAs can target Hox genes, this was the first demonstration that a miRNA could induce a homeotic segment transformation in the animal. More strikingly, *mir-iab-8* has even stronger capacity to repress *Ubx*, and correspondingly its ectopic expression generates a fuller haltere-to-wing transformation (Stark et al., 2008; Tyler et al., 2008). Notably, ectopic expression of other miRNAs with conserved seed matches in the *Ubx* 3' UTR do not generate extra wings, indicating that there is a practical difference between the existence of conserved miRNA binding sites and their ability to mediate sufficient downregulation to yield mutant phenotypes.

Another particularly compelling set of miRNA misexpression phenotypes comes from those that inhibit apoptosis or that promote tissue growth. Both of these activities are central to the process of oncogenesis and are mediated by distinct effectors, since the inhibition of apoptosis by itself does not promote tissue overgrowth. Reciprocally, promotion of cell proliferation is usually accompanied by excess cell death. Misexpression of the bantam miRNA provided early evidence for the notion of a miRNA oncogene, since this miRNA locus could both induce cell proliferation and inhibit apoptosis (Brennecke et al., 2003). miR-278 similarly exhibits joint ability to promote tissue growth and inhibit apoptosis (Nairz et al., 2006; Teleman et al., 2006), and as mentioned, miR-14 (Xu et al., 2003) and miR-263 (Hilgers et al., 2010) are also anti-apoptotic. Finally, members of the extensive K box family of miRNAs (Lai, 2002; Lai et al., 1998), the largest in *Drosophila* (*mir-2* family, *mir-6* family, *mir-11*, *mir-13* family, *mir-308*) share capacity to directly repress multiple

members of the pro-apoptotic reaper/grim/hid/sickle family (Brennecke et al., 2005). Therefore, it seems rather common amongst *Drosophila* miRNAs to be able to inhibit apoptosis and/or to promote tissue overgrowth.

These and other example from the literature indicate that despite propensity of animal miRNAs to subtly repress large numbers of targets, many *Drosophila* miRNAs elicit specific and interpretable phenotypes when misexpressed *in vivo*. Moreover, many of these effects are not predictable from target predictions. Therefore, directed phenotypic screening for miRNA gain-of-function phenotypes may be a profitable strategy to gain insight into the *in vivo* activities of miRNAs.

3.3. miRNA sponges

The improvements to HR notwithstanding, it is not a trivial effort to generate mutant alleles in *Drosophila*. Thus, an easier route towards preliminary evidence of *in vivo* miRNA function is desirable. In mammalian systems, the vast majority of loss-of-function studies rely upon modified antisense oligonucleotides, termed “antagomirs” (Krutzfeldt et al., 2005). While this method is powerful and in wide use, it is also worth considering that the a large set of antagomir-induced miRNA phenotypes in *Drosophila* (Leaman et al., 2005) were not phenocopied by subsequent null alleles. The nature of these discrepancies remain to be understood, but the possibility of off-target effects cannot be discounted. Importantly, one cannot use the lack of phenotypes induced by scrambled antagomirs, nor the apparent rescue by sense small RNAs, as compelling evidence for on-target inhibition. Although such criteria are popularly used to control mammalian studies, the former would alleviate specific off-target effects while the latter could represent a titration effect of the antagomir away from off-target substrates. Even if antagomirs are truly specific, they have limited options for tissue-specific delivery. Therefore, additional methods for miRNA sequestration are desirable.

One promising strategy is to use a decoy target transcript bearing multiple imperfect binding sites for a given miRNA, often termed a “miRNA sponge” (Figure 2B) (Ebert et al., 2007). These are proposed to act as competitive inhibitors that distract endogenous miRNAs from regulating bona fide targets. miRNA sponges have shown efficacy in lentiviral infections of mammalian cells (Gentner et al., 2009), and were recently shown to induce phenocopies of certain *Drosophila* miRNA knockouts (Loya et al., 2009). For example, transgenic sponges to miR-9a could induce posterior wing notching, as shown for the *mir-9a* deletion (Bejarano et al., 2010; Biryukova et al., 2009; Li et al., 2006), and the miR-8 sponge induce leg deformities characteristic of *mir-8* deletions (Karres et al., 2007). A caveat of these studies was that it was necessary to reduce the endogenous dosage of the “sponged” miRNA, by making the animal heterozygous for the miRNA locus, in order to obtain more consistent phenotypes (Loya et al., 2009). Potential improvement to the system may come from increasing the number of sponge sites, or the dosage of sponge transcripts.

A central feature of the Gal4-UAS system is the ability to assess autonomous and non-autonomous effects with ease. This was nicely illustrated using the miRNA sponge technique to study miR-8 function at the neuromuscular junction (NMJ). Analysis of *mir-8* mutants indicated a defect in NMJ morphogenesis (Loya et al., 2009). This might reflect an

autonomous requirement of miR-8 in CNS motor neurons, or alternatively, a non-autonomous function of miR-8 in target muscles that are innervated by these neurons. Tissue-specific expression of the miR-8 sponge showed that it was tolerated in neurons, whereas inhibition of miR-8 in muscles induced the NMJ defect, indicating that miR-8 has a non-autonomous function in controlling NMJ morphology (Loya et al., 2009).

4. Genetic interactions and epistasis tests

4.1. Double mutant analysis to uncover redundant functions

A trademark of genetic analysis is to study the consequences of combining mutations in the same organism. A few principles deserve emphasis. First is the notion of genetic interactions, that is, situations in which a given mutation exhibits a phenotype only in the background of another mutation. For example, one can imagine that null conditions for two or more genes whose loss does not result in a phenotype individually, but does in concert. Here, *C. elegans* provides the best illustrations of this principle with respect to miRNAs, owing to the large collection of extant miRNA mutants (Miska et al., 2007). For example, analysis of a triple mutant of let-7-related miRNAs (*mir-48*, *mir-84*, *mir-241*) reveals their redundant control over the transition from the second to third larval stages, via co-targeting of *hbl-1* (Abbott et al., 2005). Similarly, systematic deletion of miRNA families revealed additional examples of synthetic phenotypes (Alvarez-Saavedra and Horvitz, 2010). In *Drosophila*, a deletion of *mir-263b* did not cause obvious phenotypes; nevertheless, it could enhance the loss of eye bristles seen in the *mir-263a* knockout, implying that the function of these two miRNAs is partially redundant during eye development (Hilgers et al., 2010).

4.2. Epistasis analysis

Epistasis means “standing upon” and in a genetic sense, refers to a situation where the presence of one mutation masks the phenotype usually associated with another mutation. An epistatic relationship associated with two oppositely-directed mutants, as opposed to an intermediate phenotype of the double mutant, can provide strong evidence that two genes act in a common pathway. Moreover, the direction of epistasis can inform which gene acts upstream or downstream of the other. If mutant phenotypes are strictly epistatic, then the double mutant will resemble the single mutant of the downstream factor.

One of the most striking examples of miRNA epistasis involves bantam. As mentioned, bantam is overtly essential for proliferation of imaginal disc tissues as well as to suppress apoptosis of certain cell populations, and ectopic bantam reciprocally drives strong disc overgrowths and prevents apoptosis. Both activities are opposite to the function of the Hippo pathway, a highly conserved signaling system that restricts tissue and organ size in flies and vertebrates (Pan, 2010). In brief, a major function of Hippo signaling is to repress the activity of the Yorkie transcription cofactor. The first two transcriptional targets of Yorkie elucidated were *diap1* and *cyclinE*, which makes sense given that the former prevents cell death and the latter promotes cell cycle.

These opposite activities of Hippo signaling and bantam set the stage for a relatively clean epistatic test: what is the phenotype of imaginal disc clones that are doubly mutant for a hippo pathway member (that gives disc overgrowth) and bantam (which normally fail to

grow)? The answer is that bantam is epistatic, indicating that the disc overgrowths in hippo pathway mutant clones are driven by bantam function (Nolo et al., 2006; Thompson and Cohen, 2006). Reciprocally, constitutive activation of Hippo signaling results in apoptosis and reduced proliferation. Strikingly, this can be substantially rescued by forced activation of *bantam*, but not by *diap1* or *cyclinE* (Nolo et al., 2006; Thompson and Cohen, 2006).

Evidently, the combined activity of bantam as a pro-growth, anti-apoptotic factor define it as a key downstream target for repression by Hippo signaling. Therefore, even though it is surely the case that Yorkie has many targets genomewide, the bantam miRNA must be one of its more important effector molecules. A piece of the puzzle remains, however, since we do not yet know of any relevant targets of bantam that can explain its pro-proliferative capacity. Recall that suppression of apoptosis is not by itself sufficient to explain tissue growth (Brennecke et al., 2003). Presumably, epistatic analysis with the appropriate growth-suppressing molecules may shed light on this issue.

4.3. Rescue of miRNA mutants by target heterozygosity

Another general way to test for genetic interactions is to ask whether the mutant phenotype associated with one locus can be suppressed by modulation of another locus. In particular, cases of strong modification of one mutant by heterozygosity of another mutation can serve as compelling evidence to link the respective gene functions. This is especially the case as relatively few genes exhibit obvious phenotypes when only one allele is lost. This test, either by heterozygosity of classical mutant alleles of potential targets or by RNAi-mediated knockdown has proven to be one of the cornerstones of linking specific target genes to miRNAs in *Drosophila* (Figure 3A). As mentioned, finding “the” target gene of a miRNA is a challenging if not potentially futile task, given that most conserved miRNAs have tens if not hundreds of conserved targets. If the biological role of a miRNA is to slightly repress a hundred equivalent targets to tune the transcriptome of a given cell, then one would not expect to observe genetic interactions with any individual target. However, if there are specific targets of particular genetic importance, this situation may be fulfilled. Especially compelling would be the rescue of a miRNA mutant by heterozygosity of a given target, which would suggest that de-repression of that target plays an important role in the etiology of the miRNA mutant phenotype.

Although it might be imagined that this is an exceptional genetic situation, in fact it has proven to be rather common in *Drosophila* (Smibert and Lai, 2010). We have already mentioned how rescue of *mir-9a* and *mir-279* mutant phenotypes was achieved by target heterozygosity. Beyond these, metabolic defects in *mir-278* mutants, including insulin resistance concomitant with elevated circulating sugar, could be suppressed by heterozygosity for the FERM domain protein encoded by *expanded* (Teleman et al., 2006). NMJ morphology defects observed in a *let-7*, *mir-125* double mutants could be rescued by heterozygosity for the direct *let-7* target *abrupt*, encoding a transcription factor that regulates NMJ development (Caygill and Johnston, 2008). Additionally, the enhanced neurotransmitter release seen in the NMJ of deletion mutants of the *mir-310/311/312/313* cluster, all of which encoded seed-related miRNAs, was rescued by heterozygosity for *khc-73*, a neural-specific kinesin family member (Tsurudome et al., 2010).

These findings suggest that it may be fairly common for the de-repression of specific miRNA target genes to be of disproportionate phenotypic impact. We certainly do not wish to imply that these particular targets are the “only” targets of these miRNAs, and phenotypic rescue by target reduction is still compatible with the notion that miRNAs might commonly have hundreds of target genes. It merely highlights that these are not necessarily of equal functional regulatory consequence. This is actually reminiscent of the situation of transcription factor target genes. With modern molecular profiling methods, it is clear that typical transcription factors typically bind thousands of genomic regions (Gerstein et al., 2010; Macquarrie et al., 2011; Roy et al., 2010), and that these are often conserved (Birney et al., 2007; He et al., 2011; Stark et al., 2007). Nevertheless, it is obvious that some of these regulatory connections play more critical roles in development and physiology than many others do. It seems likely that the same consideration applies to miRNAs.

It is common for regulatory proteins to be recycled during developmental and physiological processes, and it is similarly observed that individual miRNAs function in multiple settings (Smibert and Lai, 2010). A curious emerging theme is that many miRNAs exhibit different key targets in different locations. For example, excess brain apoptosis and associated behavioral defects in *mir-8* mutants is suppressed by heterozygosity for the direct target *atrophin*, encoding a transcriptional coactivator (Karres et al., 2007). On the other hand, miR-8 also functions in the fat body to activate insulin signaling by repressing the zinc finger protein encoded by *u-shaped* (Hyun et al., 2009). Knockdown of *u-shaped*, but not *atrophin*, in the fat body rescued *mir-8* mutant phenotypes. In another example, miR-14 directly restricts the activity of the nuclear receptor encoded by the *Ecdysone receptor* during metamorphosis (Varghese and Cohen, 2007), whereas it also functions in insulin neurosecretory cells of the adult brain to directly repress *sugarbabe*, a zinc finger protein that regulates insulin gene expression (Varghese et al., 2010). It is plausible to consider that miRNAs may have so many targets, in part due to their frequent acquisition of new compelling functions in different settings via novel target genes.

4.4. Dominant modifier screens for miRNA interactors

In most cases, directed tests for genetic interactions in a homozygous mutant background requires the generation of complicated stocks or recombinant animals, which limits the throughput of analysis. However, one can exploit this test in forward genetic screening by starting with a dominant phenotype. A simple way to do this is by sensitizing the animal by misexpressing a gene to generate a mutant phenotype, and then to ask whether the removal of one copy of any other gene can modify this phenotype (Figure 3B). This approach, termed dominant enhancer/suppressor screening, was codified in studies of the Ras signaling pathway twenty years ago (Simon et al., 1991). Here, misexpression of oncogenic Ras in the developing *Drosophila* eye yielded a rough eye, presumably due to hyperactivation of its downstream pathway. Loci for which removal of one gene copy reduced the activity of oncogenic Ras identified several downstream members of the pathway (Simon et al., 1991), and helped to elucidate the signaling mechanism of this important cancer pathway. Since effects of removing only gene copy are assessed, dominant enhancer/suppressor screening can be executed on a massive scale, as illustrated by the subsequent examination of 850,000 mutagenized animals in the *Ras1* eye screen (Karim et al., 1996), which collectively hit most

of the key components of Ras signal transduction. Thus, dominant modifier screening is genuinely a “genome-wide” technique. Such an approach using chemical mutagenesis or transposon screening has yet to be applied to *Drosophila* miRNAs, but there is no particular reason to believe that it would not be profitable.

A quick-and-dirty version of the dominant screen utilizes the *Drosophila* deficiency collection, which allows one to assess genome-wide, and within only a few hundred crosses, the effects of removing one gene copy on a genetically sensitized phenotype. This strategy was recently used to great effect with the *Drosophila* ortholog of *mir-1* (King et al., 2011), a miRNA that is deeply conserved across the animals in both sequence and in specific expression in heart and muscle. Previously, misexpression of *mir-1* in the center of the developing wing, using *dpp-Gal4*, was found to generate patterning and morphogenesis defects including cell-autonomous loss of wing tissue (Kwon et al., 2005). As this presumably resulted from the ectopic repression of miR-1 target genes, such as the Notch ligand *Delta*, it was hypothesized that further reducing the dosage of relevant target genes should enhance the severity of wing phenotypes. Therefore, a *dpp-Gal4>UAS-mir-1* stock was crossed to the deficiency kit and scored for dominant enhancers that exhibited greater loss of wing tissue.

Out of 284 deficiency lines covering about 70% of the euchromatic genome, 32 enhancers were recovered (King et al., 2011). These included two conserved targets of miR-1, *Delta* and *mirror*, and novel interacting partners such as *kayak*, encoding a leucine-zipper transcription factor and a component of the planar cell polarity (PCP) pathway. Although the wing is clearly not a physiological location of miR-1 function, these interactors could be examined in the relevant tissues that endogenously express miR-1. Indeed, alteration of miR-1 dosage by null mutants or by overexpression disrupted cardioblast cell polarity in the fly heart, similar to *kayak* mutants. Importantly, heterozygosity of *kayak* could mitigate the loss of miR-1, suggesting that dysregulation of Kayak partly accounts for these defects in *mir-1* null mutants. These findings illustrate the power of *Drosophila* genetics to uncover unexpected functional connections, in an unbiased and systematic manner.

5. Detecting miRNA expression and activity in vivo

Temporal-, tissue- or cell-specificity of miRNA expression can be assessed by several strategies. These include Northern analysis or small RNA library cloning from different sources (Berezikov et al., 2011; Leaman et al., 2005; Ruby et al., 2007b), *in situ* hybridization to primary miRNA transcripts (Aboobaker et al., 2005; Kosman et al., 2004) or mature miRNAs (Li and Carthew, 2005; Sokol and Ambros, 2005). We focus this section on a few transgenic strategies that have been used to great effect in *Drosophila*, including enhancer-reporter constructs and genetically encoded miRNA sensors.

5.1. miRNA promoters “identified” by *P* elements

It is first worth commenting on the fact that *P* elements tend to insert in active promoters. Although information on miRNA promoters and transcript models from microarray analysis following knockdown of nuclear miRNA biogenesis factors (Kadener et al., 2009b), or from RNA-seq and CAGE analyses (Enderle et al., 2011; Graveley et al., 2011) is growing, our

knowledge is far from complete. It is therefore humbling to realize that *P* elements were far ahead of humans not only in identifying miRNA genes, but also their promoters. In some cases, the miRNA promoter is located quite distantly from the miRNA gene itself. For example, the *mir-278* hairpin is associated with a cluster of *P* elements located 45 kb upstream, which identifies its promoter (Nairz et al., 2006; Teleman et al., 2006). Similarly, the *bantam* miRNA hairpin is associated with multiple *P* element insertion clusters, which likely signify complexity of its transcriptional control via multiple promoters; the most distal promoter is some 40 kb away from the *bantam* hairpin (Graveley et al., 2011; Peng et al., 2009). Importantly, *P*-elements that contain marker genes can report on the transcriptional output of the promoter within which they are inserted (Figure 4A) through expression of the marker gene (e.g. *bereft/mir-263a:lacZ*, Section 2.2). This is particularly useful for miRNA genes, as it provides a relatively stable readout of primary transcript expression of the tagged miRNA locus.

5.2 miRNA enhancer:reporter transgenes

“Enhancer bashing” is a time-honored tradition in *Drosophila*, an exercise in which one attempts to identify fragments of genomic DNA that can drive a reporter gene (e.g. *lacZ*, *GFP*, *DsRed* etc) in a pattern that recapitulates known aspects of the endogenous transcript (Figure 4A). An *in situ* hybridization survey of primary miRNA transcripts revealed a diversity of spatially restricted patterns in *Drosophila* embryos (Aboobaker et al., 2005), and relevant *cis*-regulatory modules (CRMs) for several embryonically expressed miRNAs have been isolated, including for the *mir-309/3/286/3/4/5/6* cluster in the early blastoderm embryo (Biemar et al., 2005), for *mir-1* in the mesoderm and muscles (Biemar et al., 2005; Kwon et al., 2005; Sokol and Ambros, 2005) and for *mir-124* in the central nervous system (Xu et al., 2008). Similarly, a *mir-7* CRM located within the intron of its host transcript *bancal* (Li and Carthew, 2005; Li et al., 2009) and a CRM upstream of *mir-279* (Cayirlioglu et al., 2008) have proven useful to probe post-embryonic expression of these miRNAs in the nervous system. In many of these examples, relevant binding sites for transcription factors have been elucidated. For example, analysis of *mir-1* CRMs indicated direct regulation by the transcription factors Dorsal, Twist, d-Mef2, and potentially SRF (Biemar et al., 2005; Kwon et al., 2005; Sokol and Ambros, 2005).

Even without prior knowledge of an endogenous transcript pattern, reporters can provide an entry point to elucidate spatial patterns. In this case, a critical concern is to include sufficient regulatory sequence to encompass relevant inputs. This might be addressed by inserting reporter sequences into large constructs (such as fosmids or BACs), or better yet, by inserting within the endogenous locus. One way this can be accomplished is to include a reporter, such as Gal4, within an HR targeting cassette. Combining a UAS-reporter with a Gal4 knock-in allele was first used to demonstrate that *mir-278* was highly active in the fat body, where it regulates energy homeostasis by limiting insulin pathway activity and levels of circulating sugar (Teleman et al., 2006). Subsequent integrations of Gal4 into the *let-7/mir-100/mir-125* locus (Sokol et al., 2008) and the *mir-263a* and *mir-263b* loci (Hilgers et al., 2010) have been useful to report on their expression, as well as to manipulate gene expression within miRNA-expressing cells.

5.3 Genetically encoded sensors of miRNA activity

The above methods have collectively been powerful in analyzing miRNA expression in the *Drosophila* system. However, one potential limitation is that these strategies do not directly report on miRNA activity. Given the growing appreciation of post-transcriptional regulation of miRNA processing and/or function (Kim et al., 2010; Siomi and Siomi, 2010), it is useful to be able to monitor miRNA activity *in vivo*. Such an approach was pioneered eight years ago with the bantam miRNA (Brennecke et al., 2003). The bantam “sensor” transgene consisted of a ubiquitously expressed GFP transcript with several perfectly complementary bantam sites in its 3' UTR (Figure 4B); thus, the expression of GFP is lowest where the activity of bantam is highest (Figure 4C). The bantam sensor exhibits spatially modulated activity in imaginal discs, reflecting its function in growth regulation.

The bantam sensor has been widely exploited as a proxy readout of its response to various signaling pathways, presumably at the transcriptional level. For example, a complement to the aforementioned epistatic studies tests between upstream Hippo pathway members and bantam was to examine the bantam sensor in Hippo pathway mutant clones. These tests revealed lower bantam sensor levels in these clones in wing imaginal discs, indicating increased bantam activity (Nolo et al., 2006; Thompson and Cohen, 2006). The bantam sensor was later shown to be upregulated by activation of Notch signaling in a particular region of the wing disc (Herranz et al., 2008), suggesting a repressive input of Notch pathway activity onto bantam. Finally, the bantam sensor helped reveal a direct input of the Homothorax transcription factor, in complex with Yorkie, that drives proliferation in the eye imaginal disc (Peng et al., 2009).

Most recently, exploitation of the bantam sensor uncovered a novel and direct intersection of the Hippo and TGF- β signaling pathways that drives bantam expression in both wing and eye discs (Oh and Irvine, 2011). Indeed, protein-protein interactions between the mammalian Yki ortholog Yap and TGF- β pathway Smad transcription factors have been documented (Alarcon et al., 2009; Ferrigno et al., 2002), although the *in vivo* consequences of this for Hippo signaling or TGF- β signaling were not known. Taking a cue from the essential role for cell signaling via the *Drosophila* TGF- β ligand Dpp for tissue growth and patterning (Affolter and Basler, 2007), it was found that coexpression of Yorkie and with an activated Dpp receptor (Tkv^{QD}) synergistically promoted tissue growth and repression of the bantam sensor, indicating increased bantam activity. Reciprocally, blocking Dpp pathway activity increased bantam sensor expression, indicating repression of bantam function (Martin et al., 2004; Oh and Irvine, 2011). (Note that these experiments involved keeping mutant cells alive using the anti-apoptotic factor p35, since cells are otherwise succumb without Dpp signaling).

These observations laid a foundation for multifaceted studies involving many other principles we have discussed in this review. First, epistatic tests (Section 4.2) showed that forced expression of bantam could partially rescue the failure of cells lacking Dpp signaling to survive and proliferate. Second, enhancer bashing (Section 5.2) identified a bantam CRM that was synergistically responsive to Yorkie and Dpp pathway function in imaginal discs.

Finally, it was shown that a novel transcriptional complex containing Yorkie and the Dpp pathway transcription factor Mad directly bind and activate this bantam CRM.

Altogether, this collection of studies of the bantam sensor helped reveal an extraordinary diversity of transcriptional inputs into this miRNA. Such complexity befits an essential growth locus such as bantam, presumably to coordinate precise tissue growth underlying appropriate development. Interestingly, bantam has additional roles independent of growth, such as in scaling growth of neural dendrites (Parrish et al., 2009), maintenance of germline stem cells (Yang et al., 2009) and control of circadian rhythm (Kadener et al., 2009a). Presumably each of these other settings is associated with other transcriptional inputs to bantam, which remain to be elucidated. Finally, while the bantam sensor is the most well-studied in *Drosophila*, other sensors for individual miRNAs [e.g. miR-9a (Bejarano et al., 2010) or miR-14 (Varghese et al., 2010)] or for miRNA-regulated 3' UTRs (Figure 4B) (Bejarano et al., 2010; Brennecke et al., 2003; Friggi-Grelín et al., 2008) have been informative probes of miRNA activity *in vivo*. In many post-embryonic tissues, one can induce clones that are homozygous for a mutant allele of interest. In combination with a miRNA sensor, induction of clones of a miRNA mutant can report on the activity of that miRNA in a particular tissue; de-repression of a miRNA or 3' UTR sensor reports directly on miRNA activity (Figure 4C).

6. Conclusions and Future prospects

We have emphasized a broad selection of *Drosophila* genetic techniques used to manipulate miRNA activity, elucidate associated pathways and key target genes, and interrogate miRNA expression, all in the context of the intact animal. Overall, we wish to highlight that miRNAs are just like any other genes, in that they can be studied using most of the same techniques used to study “interesting” protein-coding genes. Although the field of miRNA biology is dominated by reverse genetics, many miRNAs are associated with palpable phenotypes, both loss- and gain-of-function. However, it can take some searching to find the right place, the right time, and the right markers with which to characterize miRNA activities. On the other hand, we also highlight that manifestations of miRNA dysfunction have existed in *Drosophila*, as with *C. elegans*, long before the formal recognition of miRNAs as a unified class of regulatory molecule. We conclude with a few final points with a view to the future of miRNA genetics in *Drosophila*.

First, we are struck by the fact that overexpression of so many miRNAs induces compelling and interpretable phenotypes. This is not necessarily expected from the viewpoint that animal miRNAs predominantly mediate subtle repression of large groups of transcripts, which might imply that manipulation of miRNAs might either yield few phenotypes, or alternatively elicit non-specific toxic effects caused by simultaneous misregulation of hundreds of transcripts. As further examples, misexpression of many miRNAs elicit specific developmental phenotypes reflecting gain- or loss-of-function of the core cell signaling pathways (Hagen and Lai, 2008), such as Notch (Lai et al., 1998; Lai and Posakony, 1997; Lai et al., 2005; Stark et al., 2003), Hippo (Brennecke et al., 2003), Hedgehog (Friggi-Grelín et al., 2008), Wnt (Silver et al., 2007). Efforts are underway to extend plasmid-based UAS-miRNA libraries used to screen tissue culture cells (Silver et al., 2007) into transgenes, for

systematic *in vivo* phenotypic screening in the animal (Y. Chou, F. Bejarano, D. Bortolamiol-Becet and E. C. Lai, unpublished). We anticipate that a similar set of resources in the mouse would have a high probability of revealing a great diversity of disease-relevant miRNA activities, most of which would probably not be easily anticipated from extensive lists of miRNA target predictions.

Conversely, the generation and analysis of more miRNA deletion alleles is heavily anticipated. In contrast to the *C. elegans* system, where very few miRNA mutants have overt phenotypes (Miska et al., 2007), even when examining multiple mutants of entire families (Alvarez-Saavedra and Horvitz, 2010), the extant literature indicates an impressive set of developmental and physiological defects associated with many different *Drosophila* miRNA mutants, under non-perturbed conditions. Perhaps this reflects the rich phenotypic assays available in *Drosophila*, and we suspect that mammalian systems (e.g. mice and humans) are similarly phenotypically rich. It is worth considering that many seemingly inert *C. elegans* miRNA deletions generate phenotypes in genetically sensitized backgrounds (Brenner et al., 2010). Similarly, environmental stress can strongly enhance the phenotypes of the *Drosophila mir-7* mutant (Li et al., 2009). Therefore, future miRNA deletion studies in flies should take advantage of both normal and sensitized backgrounds.

The flexibility of manipulating endogenous miRNA loci will be greatly increased by recent upgrades to HR technologies. These include the development of “ends-out” targeting for direct allele replacement (Gong and Golic, 2003) and various modifications that improve the ease and efficiency of detecting candidate targeting events (Huang et al., 2009). Even more valuable are the development of “genomic engineering” strategies to permit rapid construction of allelic variants using a founder line modified to contain phage phiC31 integrase recognition sites (Figure 1F) (Choi et al., 2009; Gao et al., 2008; Huang et al., 2009). This is probably the way forward for generation of reverse-engineered alleles in *Drosophila*, including miRNAs (Weng et al., 2009). For example, one can imagine generating one founder mutant allele that deletes a miRNA locus, then quickly using that as a platform to reintroduce the miRNA to demonstrate rescue of the targeted allele, to insert a nuclear enhancer trap marker, a membrane-localized marker (for example, useful with neural genes to delineate their projections), a Gal4 driver line to express other genes in the miRNA pattern, to insert variant versions of the miRNA sequence for structure-function tests, and so forth. The near future should see broad utilization of these powerful technologies.

Finally, we envision that the ultimate proof of critical miRNA:target interactions will involve assessment of target transgenes specifically mutated for miRNA binding sites. In fact, such evidence was generated in *Drosophila* in the “pre-miRNA” era, with the observation that genomic transgenes for Notch pathway members *Bearded* and *E(spl)m8* specifically mutated for Brd box and K box miRNA binding sites generated dominant gain-of-function phenotypes in the nervous system (Lai et al., 1998; Lai and Posakony, 1997), echoing the dominant alleles involving 3' UTR aberrations that permitted the cloning of these genes (Klambt et al., 1989; Leviten et al., 1997). Similarly, the very recognition of the miRNA pathway depended in part on the existence of *lin-14* gain-of-function mutants caused by 3' UTR deletions that removed *lin-4* binding sites (Ruvkun and Giusto, 1989;

Wightman et al., 1991). Although there is good evidence that a large fraction of the *Drosophila* transcriptome is regulated by highly conserved miRNA binding sites (Ruby et al., 2007b), it is difficult to imagine that the specific loss of these regulatory interactions will result in noticeable phenotypes. Greater knowledge of some of these “critical” targets, for which loss of miRNA-mediated regulation is associated with developmental or physiological abnormalities, will not only be interesting to insect fanciers, but should be valuable for understanding how miRNA dysfunction contributes to human disease.

Acknowledgments

Q.D. was supported by a fellowship from the Swedish Research Council. Work in E.C.L.’s group was supported by the Burroughs Wellcome Fund, the Alfred Bressler Scholars Fund, the Starr Cancer Consortium (I3-A139) and the NIH (R01-GM083300).

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Modes of mutagenesis in Flies (as applied to miRNA loci)

Figure 1. Making fly miRNA mutants

The major methods for generating mutations in fly miRNA loci are described. **A.** Chemical mutagens, though very useful for generating mutant alleles of protein-coding genes, have been of limited use in investigating *Drosophila* miRNA genes. The only published example is a point mutant in miR-278 that was recovered as a revertant of a gain-of-function phenotype (see Section 3.1). **B.** Transposon insertions can interfere with transcription and therefore have potential to be mutagenic for miRNA loci. The *P* element strong prefers to insert in promoters, and can disrupt transcription. The *Minos* and *piggyBac* elements, whose

insertion preferences are less biased, can disrupt miRNA primary transcripts. **C.** *P* elements (and to a lesser extent *Minos* elements, but not *piggyBacs*) can be used to generate local deletions by imprecise excision. **D.** FRT-mediated deletion. FLP recombinase can induce deletions between different FRT-containing transposons located *in trans*. This can result in the precise deletion of the intervening region. **E.** Homologous recombination (HR) allows the precisely engineered generation of a mutant allele. The miRNA hairpin can be replaced with a sequence of choice. **F.** Recently, the advent of genomic engineering has made it possible to generate a founder knockout line by HR that contains a phage attachment site (not shown) which enables relatively easily modification of the founder line as desired, for example adding various markers, Gal4 transgenes or modified hairpins to the endogenous locus.

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A. Ectopic expression of miRNAs

- i. EP elements : P-element with UAS sites for Gal4-mediated activation

- *Misexpression of endogenous miRNA locus*
- *Different Gal4 lines provides tissue specificity*

- ii. UAS-miRNA transgenes

- *Engineered construct*
- *Can include marker transgene (eg DsRed)*

B. Expression of miRNA sponges

- *Sequester miRNA from endogenous targets*
- *Creates partial loss-of-function*
- *Can include marker transgene (eg GFP)*

Figure 2. Modulating miRNA activity using the Gal4/UAS system

Gal4/UAS is a modular system for overexpressing transcripts under the control of the yeast Gal4 transcription factor, via “upstream activating sequences” (UAS) linked to a target gene. Many spatially/temporally-restricted Gal4 transgenes are available. **A.** Ectopic expression of miRNAs. As with protein coding genes, ectopic expression of miRNAs can lead to dominant phenotypes. Many miRNA loci were discovered by genetic screens of collections of EP elements (**i**) - transposons bearing UAS sites, which permit activation of neighboring genes in a Gal4-dependent manner. Engineered UAS-miRNA transgenes (**ii**) often contain the

hairpin flanked by ~200 nt of endogenous sequence, as well as a marker transgene that reports on miRNA-expressing cells. **B.** Inducible miRNA sponge transgenes, bearing multiple copies of a given miRNA target site, enable tissue-specific knockdown of miRNA activity.

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A. Suppression of miRNA loss-of-function phenotype

B. Enhancement of miRNA gain-of-function phenotype

Figure 3. Usage of genetic interactions to elucidate miRNA function

Since miRNAs act by repressing target genes, the phenotypic outcome of modulating miRNA activity can be sensitive to the dose of the key miRNA target(s). **A.** In a miRNA loss-of-function condition, crucial target(s) are de-repressed, leading to a phenotype. Lowering the dose of these target(s) by heterozygosity or RNAi can reduce the target levels to below a phenotypic threshold and suppress the phenotype. This situation can only be fulfilled when there are individual target genes that contribute substantially to organismal phenotype; nevertheless, this seems to be common amongst *Drosophila* miRNA mutants. **B.**

In a miRNA gain-of-function condition, a phenotype can arise due to crucial target(s) being lowered to levels below a phenotypic threshold. Heterozygosity, or RNAi knockdown of these target(s) can enhance this phenotype. Note that for both loss- and gain-of function miRNA conditions, the observation of genetic interactions with target heterozygosity is more powerful than the usage of RNAi transgenes. Target knockdown often generates phenotypes on its own, whereas heterozygosity is usually phenotypically benign.

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A. miRNA expression reporters

i. Enhancer traps

- Report transcriptional output of endogenous miRNA locus

ii. Direct enhancer : reporter transgenes

- Report activity of one or more miRNA enhancers

B. miRNA activity sensors

i. miRNA “sensor” transgenes

- Multimer of perfect complementary sites to a miRNA
- Reports on miRNA function

ii. 3' UTR reporters

- 3' UTR of miRNA-regulated gene
- May be regulated by several miRNAs

C. Use of miR sensors / reporters

i. Ectopic miRNA test

- Repression of reporter (green) in miRNA-expressing domain (red)

ii. Loss of miRNA test

- Induction of homozygous miRNA mutant (-/-) and wildtype (+/+) clones in heterozygous animal (+/-)
- De-repression of miRNA sensor mutant cells

Figure 4. Reporters of miRNA expression and activity

miRNAs are transcribed as long primary transcripts, whose mature ~22 nt products guide Argonaute proteins complexes to target transcripts. Note that the transcription of a miRNA locus does not necessarily report directly on cells in which the miRNA is activity. **A.** miRNA expression reporters are the same as those used to report expression of protein-coding genes. **(i)** Putative regulatory sequences are identified by enhancer traps (insertion of transposon with a marker gene, often *lacZ* or *Gal4*) where the endogenous *cis*-regulatory modules (CRMs) drive expression of the marker. **(ii)** Cloned CRMs can also be used to

analyze miRNA gene expression; however, these may only report on a subset of the full transcriptional control of a miRNA. **B.** Sensors of miRNA activity typically use a marker gene (often *GFP*) bearing sequences complimentary to the miRNA of interest **(i)** or with the 3' UTR of a gene of interest **(ii)** cloned downstream. Sensor activity is inverse to that of the miRNA: low sensor expression equates to high miRNA activity and *vice versa*. **C.** Schematic representation of *Drosophila* wing imaginal discs, illustrating how miRNA reporters and sensors reflect miRNA activity in overexpression and loss-of-function scenarios. **(i)** Overexpression of the miRNA in a defined stripe of cells (red) leads to cell-autonomous repression of the reporter (green); neighboring non-miRNA-expressing cells serve as an internal control for the experiment. **(ii)** Mutant clones of a miRNA (negatively marked by absence of red staining) derepress the miRNA sensor (green), indicating loss of an endogenous miRNA:target repression event. Homozygous mutant ($-/-$) and wild-type cells ($+/+$) are generated within an otherwise heterozygous animal. Again, cell-autonomy of the sensor de-repression, provides stringent controls for the experiment.