Review TRENDS in Genetics Vol.20 No.12 December 2004

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MicroRNAs and the regulation of cell death

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Programmed cell death, or apoptosis, is ubiquitous, both during development and in the adult. Many components of the evolutionarily conserved machinery that brings about and regulates cell death have been identified, and all of these are proteins. However, in the past three years it has become clear that roughly 1% of predicted genes in animals encode small noncoding RNAs known as microRNAs, which regulate gene function. Here we review the recent identification of microRNA cell death regulators in *Drosophila*, hints that such regulators are also likely to exist in mammals, and more generally the approaches and tools that are now available to probe roles for noncoding RNAs in the control of cell death.

Apoptotic cell death is an evolutionarily conserved process by which organisms remove cells that are superfluous, that have outlived their usefulness, or that are dangerous for the survival of the organism. Deregulation of cell death is associated with many human diseases. Cancer, and autoimmuity resulting from a defective removal of self-reactive T-cells, are associated with decreased cell death, whereas neurodegenerative diseases and AIDS are associated with increased cell death (reviewed in Refs [1–3]). Essentially all of the identified cell death regulatory genes encode proteins. Genetic and biochemical screens for protein-encoding death regulators made sense given what we knew at the time – that proteins are the primary effectors and regulators of cellular functions. However, within the past several years it has become clear that genomes of plants and animals encode hundreds of small noncoding RNAs known as microRNAs (miRNAs). Roughly 30% of these miRNAs are highly evolutionarily conserved. Although the functions of most are unknown, it is already clear that miRNAs are involved in the regulation of many different processes (reviewed in Refs [4–6]).

We [7], as well as the Cohen laboratory [8], recently identified several *Drosophila* miRNAs that encode potent cell death inhibitors. These observations are important because they define new points and mechanisms of cell death regulation. In addition, they suggest a heretofore hidden resource of cell death regulators whose deregulation might contribute to human disease. For example, death-inhibiting miRNAs, being very small and noncoding, make for difficult-to-hit targets in genetic screens; moreover, biochemical screens for death regulators have not been designed to identify small RNAs. miRNAs would also have been missed in experiments designed to identify candidate oncogenes or tumor suppressors through transcriptional profiling of normal and transformed cells because these experiments did not include these molecules.

miRNAs, their abundance and mechanisms of action

The first miRNAs to be discovered, *lin-4* and *let-7*, were identified genetically, based on their roles in the development of *Caenorhabditis elegans* (reviewed in Ref. [9]). More recently it has become clear that *lin-4* and *let-7* are the founding members of a large class of small regulatory RNAs. Multiple groups, using direct cloning of small RNAs, biochemical purification of ribonucleoprotein (RNP) particles, and computational approaches that involve searches for evolutionarily conserved stem-loop structures characteristic of miRNA precursors, have identified a large number of small noncoding RNAs (roughly 21–23 nucleotides). The microRNA Registry (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml) provides an up-to-date listing of miRNAs. It is currently estimated that the genomes of complex organisms such as plants, worms, flies and humans contain some several hundred miRNAs, ≈1% of currently predicted genes (reviewed in Refs [4,5]). However, this estimate relies heavily on the assumption that all miRNAs share a certain degree of structural similarity with those already identified. If this constraint is relaxed, the actual number of miRNA-encoding genes could be significantly larger.

The reader is referred to several recent reviews for detailed discussions of the biology and function of miRNAs and related small RNAs [4,5,10]. Here and in Figure 1 we summarize several important points. Many miRNAs are developmentally regulated at the transcriptional level. Some are transcribed as free-standing genes. Others are made as polycistronic transcripts that encode multiple miRNAs, and still others are found in introns of protein-coding genes, often pointing in the same direction as the primary transcript. The primary miRNA-encoding transcript is known as a pri-miRNA, and can be long. In animals (but not in plants, which utilize other proteins) this transcript is processed in the nucleus by the nuclease Drosha, to generate a ≈60–80 nucleotide stem-loop

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Available online 8 October 2004

 0168-9525/$ - see front matter © 2004 Elsevier Ltd. All rights reserved. doi:10.1016/j.tig.2004.09.010

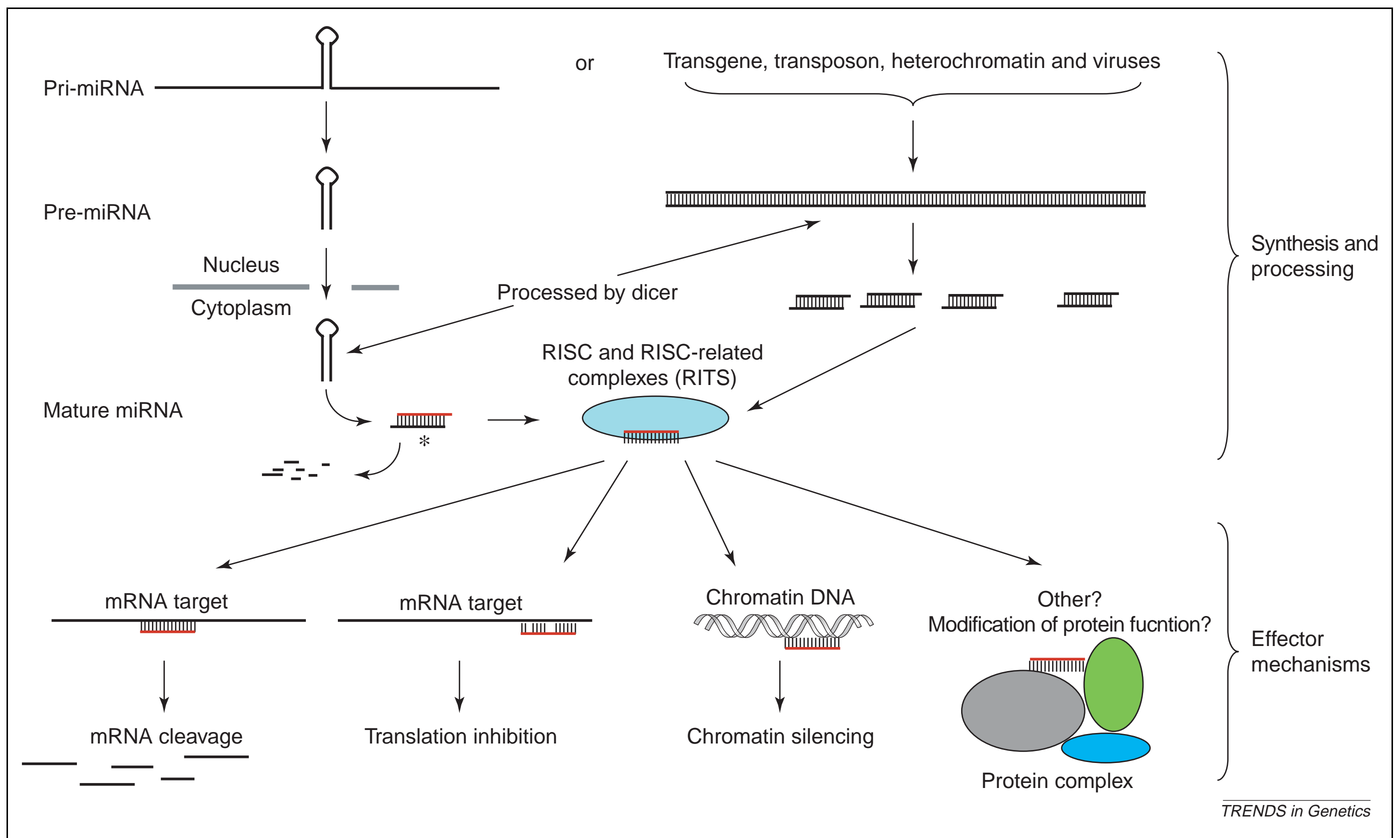

Figure 1. A simplified model for the processing and functions of microRNAs (miRNAs) and some other small RNAs (siRNAs). The focus is on the processing and roles of the siRNAs. For each of the steps indicated, one or more proteins (not shown) are also required. miRNA biogenesis is shown on the left and siRNA biogenesis is shown on the right. See the main text for details of miRNA synthesis and processing. MiRNA–siRNA duplexes resulting from Dicer cleavage are incorporated into one or more versions of a multiprotein complex known as the RNA-induced silencing complex (RISC). During this process the duplex is unwound. One strand, known as miRNA*, is degraded, whereas the other strand (indicated in red) is retained within the RISC. This strand is thought to act as a guide to identify targets for RISC action. High levels of complementarity to an mRNA (which need not be 100%) [53,54], result in cleavage of the substrate mRNA between nucleotides corresponding to residues ten and 11 of the miRNA, miRNA, as is seen with siRNA-guided cleavage. Lower levels of complementarity following rules that are just emerging (see main text), specify translational repression. Importantly, the method by which RNA-loaded RISC complexes repress gene function depends not on the origin of the RNA within the cell – as a siRNA or a miRNA – but on the level of homology with the target (reviewed in Ref. [4]). Finally, it is important to mention that siRNAs, functioning as a part of RISC-like complexes, can also regulate gene function through other mechanisms. These include the promotion of DNA methylation and gene silencing in plants, heterochromatin formation in fungi and DNA rearrangements in ciliates (reviewed in Refs [4,10]). Components of the RNA interference (RNAi) pathway have been demonstrated to have roles in heterochromatin-mediated gene silencing in *Drosophila* [55], and in fission yeast a RISC-related complex (known as RITS) has been identified that probably mediates RNAi-dependent chromatin modification and silencing (reviewed in Ref. [10]). miRNAs have not been shown to enter the RITS complex or to participate in chromatin silencing, but given the early stage within the field, it seems premature to conclude that they never have such roles. By a similar logic, neither miRNAs nor siRNAs have been demonstrated to alter the function of other cellular proteins or protein complexes. However, the recent observation that small RNAs can modify protein function in mammals [56] argues that we should remain vigilant to the possibility of other modes of action.

intermediate known as a pre-miRNA. The Drosha cleavage site defines one end of the mature miRNA product. The pre-miRNA is actively transported out of the nucleus where it is further processed in the cytoplasm by the nuclease Dicer, which also participates in the generation of small interfering RNAs (siRNAs) that mediate RNA interference (RNAi). Dicer cleavage occurs at a fixed (21–22 nt) distance from the end generated by Drosha, generating a siRNA-like duplex. One arm of this, the miRNA, becomes incorporated into one or more versions of a multiprotein complex known as the RNA-induced silencing complex (RISC), whereas the other arm, the miRNA*, is degraded. The choice of which strand enters the RISC, for both miRNAs and siRNAs, is largely determined by the stability of the 5′ end: the strand that enters the RISC is usually the one that is most weakly paired at its 5′ end [11,12]. RNA-loaded RISC can repress gene function in several different ways, as outlined in Figure 1.

The *Drosophila* cell death machine

The core of the cell death machine in animals consists of members of a family of proteases known as caspases (reviewed in Ref. [13]). Caspases become activated in response to many different death signals. Active caspases then cleave several different cellular substrates, ultimately leading to cell death and corpse phagocytosis. Most if not all cells constitutively express caspase zymogens (inactive precursors) sufficient to bring about apoptosis Thus, the key to cell death and survival signaling revolves around controlling the levels of active caspases in the cell. Following activation, caspase activity is antagonized by the action of members of the IAP (inhibitor of apoptosis) family of cell death inhibitors, the only known cellular caspase inhibitors (reviewed in Ref. [14]). Important components of the mammalian and *Drosophila* cell death machines and sites of miRNA action discussed in the text are illustrated in Figure 2 and discussed in the legend.

mir-14 *is a dose-dependent inhibitor of cell death and fat storage*

The cell death inhibitor DIAP1, the product of the *thread* (*th*) locus, has a central role in the regulation of cell death in *Drosophila* (reviewed in Ref. [14]). We originally identified DIAP1 as a cell death suppressor in a screen for loss-of-function mutations that when heterozygous enhanced cell death induced by eye-specific expression of Rpr or Hid [15]. We also identified DIAP1 several years later in a screen for genes that suppressed cell death when they were ectopically expressed in response to nearby insertion of a *P* element vector (GMREP) that carries an eye-specific promoter near one end [16]. These early screens did not proceed to saturation. Therefore, we extended them in a search for new cell death suppressors. We screened available *P* element insertion collections (presumably decrease or loss of function) for enhancers of Rpr-, Hid- or Grim-overexpression-dependent cell death in the eye. We also generated and screened ≈8000 insertions of the GMREP vector (Figure 3) for suppression of Rpr-, Hid- or Grim-dependent death and identified 11 loci associated with strong cell death suppression ([7,17], P. Xu and B. Hay, unpublished). One region of the second chromosome, at 45F1, was particularly intriguing because *P* element insertions from both screens were found to be clustered within a small 3-kb region (Figure 3). Together, these observations strongly suggested the existence of a nearby cell death inhibitor. However, none of these insertions affected the expression (as determined by *in situ* hybridization) of nearby (or not so nearby) protein-coding genes in the then-available annotations of the *Drosophila* genome.

The answer to this puzzle became clear (after more years then we care to recall) when Tuschl and colleagues reported the first cloning of *Drosophila* miRNAs [18]. All of the 45F1 *P* elements identified in both screens for death suppressors were located 5′ to a miRNA known as *mir-14*. Eye-specific expression of a small 118-bp fragment of genomic DNA that contained *mir-14* acted as a potent suppressor of death induced by expression of the cell death activators Rpr, Hid, Grim or the apical caspase Dronc, and a small deletion (*mir-14*$^{\Delta 1}$; 533 bp) that removed *mir-14* resulted in cell death enhancement [7] (Figure 3). Importantly, this latter phenotype as well as other phenotypes associated with loss of *mir-14* (discussed in the following section) was suppressed in the presence of a small genomic rescue fragment of 3.4 kb that contained *mir-14*, but no other known genes. Together these observations show that *mir-14* is a dose-dependent cell death suppressor.

Many individuals lacking *mir-14* die during the pupal stages. Those that do survive are stress sensitive and have

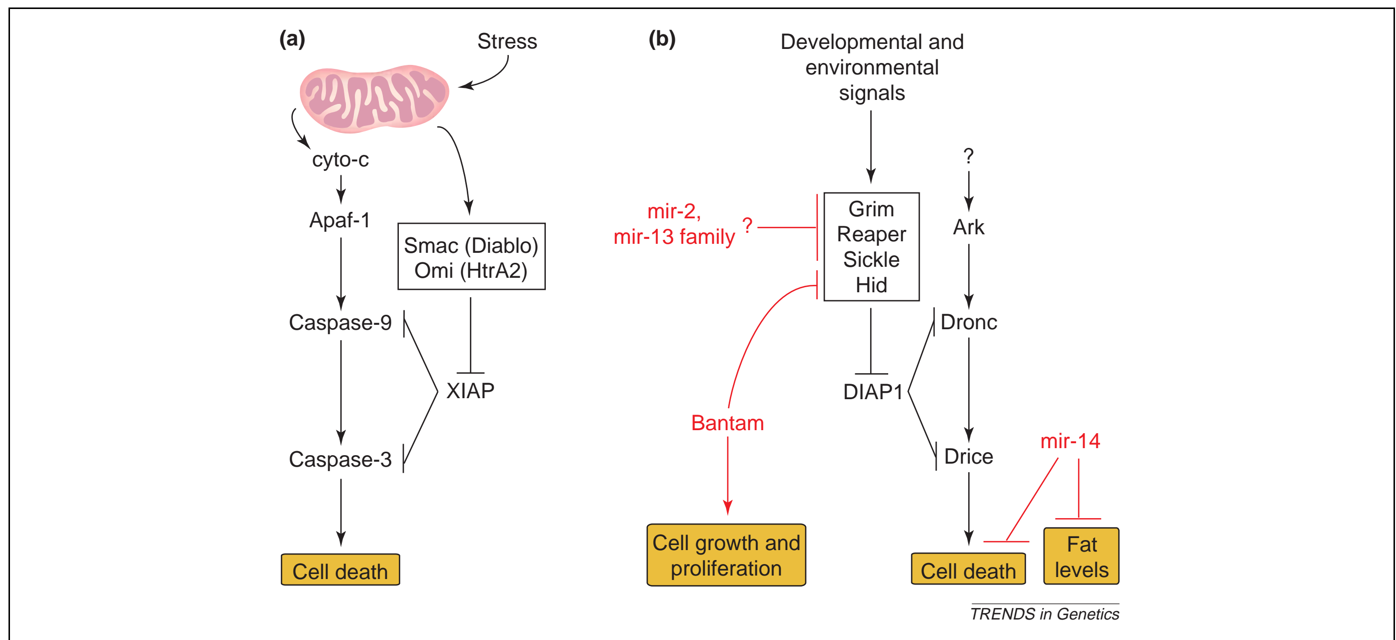

Figure 2. Proteins, microRNAs and the cell-death machine. **(a)** A major pathway by which caspase-dependent cell death occurs in mammals. **(b)** A major pathway by which caspase-dependent cell death occurs in *Drosophila*. In mammals, various forms of cellular stress lead to mitochondrial damage, resulting in the release of proapoptotic proteins, including cytochrome *c*, Smac (Diablo), and Omi (HtrA2). Cytochrome *c* binding to Apaf-1 promotes Apaf-1-dependent activation of Caspase-9, which itself cleaves and activates effector caspases such as Caspase-3. Caspase activity is inhibited by inhibitor of apoptosis (IAP) family members such as XIAP, which are themselves inhibited by Smac (Diablo) and Omi (HtrA2) (reviewed in Ref. [57]). In *Drosophila*, many cells, including those that normally live, experience chronic activation of the upstream cell death caspase Dronc, mediated by the adaptor Ark (Ark is the official Flybase name; also referred to as HAC-1, dApaf-1 and Dark), the *Drosophila* Ced-4 (Apaf-1) homolog [58–61]. Currently, it is unclear if Ark activity is itself regulated, indicated by the question mark ('?'). If unrestrained, active Dronc cleaves and activates downstream effector caspases such as Drice that mediate cell death. In the face of continuous Dronc activation, cell survival requires DIAP1, which suppresses Dronc activity (and the activity of caspases activated by Dronc) (reviewed in Ref. [14]). Expression of Reaper (Rpr), Head involution defective (Hid), Grim and/or Sickle is induced in cells targeted for death. These proteins promote death, at least in part, by disrupting DIAP1–caspase interactions through several different mechanisms, each of which has the effect of unleashing a cascade of apoptosis-inducing caspase activity. Proteins that perform proapoptotic functions analogous to those of Rpr, Hid, Grim and Sickle are present in mammals [Smac (Diablo) and Omi (HtrA2)], but not in *Caenorhabditis elegans*, which also lacks identified caspase inhibitors. Several known or suspected noncoding miRNA cell death inhibitors are also illustrated. The *Drosophila* miRNA *bantam* suppresses Hid translation. *bantam* also promotes cell growth and proliferation through action on unknown targets. *mir-2* and *mir-13* family members can inhibit translation of transcripts that contain predicted target sites found in transcripts of *rpr*, *grim* and *sickle*. This suggests but does not prove that they can inhibit Rpr-, Grim- and Sickle-dependent death *in vivo*. The provisional nature of this conclusion is indicated by the question mark ('?'). *mir-14* is a dose-dependent suppressor of fat storage and cell death whose targets are unknown.

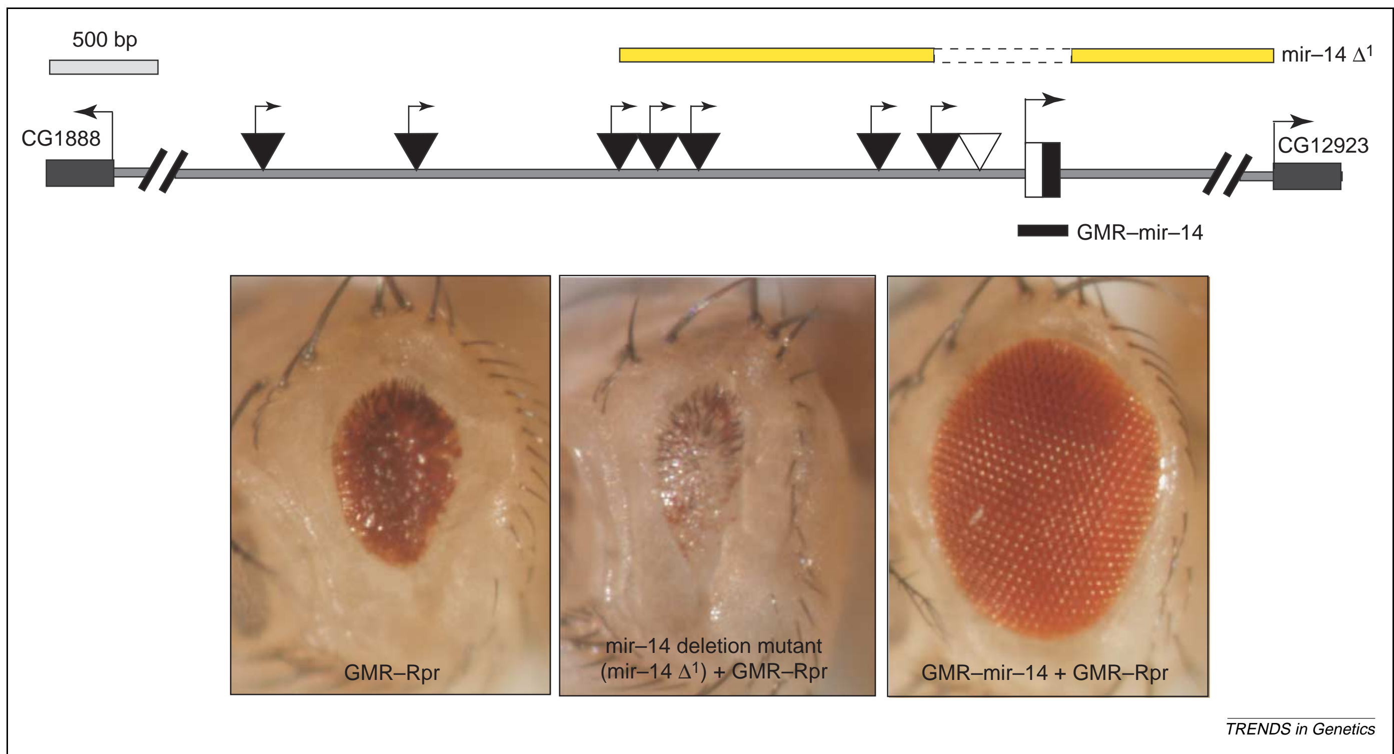

Figure 3. The cell death suppressor *mir-14*. The upper half of the figure shows the *mir-14* genomic region. GMREP is a *P* element transposon that contains an eye-specific promoter near one end, pointing out into the surrounding genomic DNA where it can drive the expression of nearby genes. Filled triangles with arrows indicate the positions of GMREP elements identified as suppressors of ectopic Reaper (Rpr) expression in the fly eye (GMR–Rpr flies). The open triangle indicates the position of the *P* element insertion identified in the loss-of-function screen as an enhancer of GMR–Rpr-dependent cell death. The positions of the *mir-14*$^{\Delta 1}$ deletion and the 118 bp fragment of genomic DNA containing *mir-14* expressed under GMR-control (GMR–mir–14) are also indicated. The lower half of the figure shows pictures of fly eyes of various genotypes. From left to right these are: GMR–Rpr; GMR–Rpr flies heterozygous for the *mir-14*$^{\Delta 1}$ deletion; and GMR–Rpr flies that also express GMR–mir–14. Expression of Rpr in the eye results in flies that have small eyes. This small eye phenotype is enhanced by a loss of half the endogenous dose of *mir-14*, and is completely suppressed by eye-specific expression of *mir-14*. Adapted, with permission, from Ref. [7].

a short lifespan. These phenotypes might result from loss of *mir-14* in its capacity as a cell death inhibitor. Consistent with this possibility, levels of the cell death effector caspase Drice are elevated in *mir-14* mutants [7]. However, *mir-14* flies also have a second set of intriguing phenotypes that complicates this interpretation. They are obese, with roughly twice the levels of triacylgycerol (TAG) compared with wild-type animals. Levels of diacylglycerol (DAG), but not those of other lipids, are also elevated. Increasing *mir-14* levels resulted in a converse set of phenotypes – decreased TAG and DAG levels. In fact recently we have observed that expression of *mir-14* specifically in the *Drosophila* fat body (the equivalent of our adipose tissue) can drive total adult TAG levels down to roughly a fifth of their normal levels (P. Xu and B. Hay, unpublished). Thus, *mir-14* is also a potent dose-dependent regulator of fat storage and/or metabolism.

Is there a relationship between the effects of *mir-14* on cell death and TAG and DAG levels? Several links between fat metabolism, TAG, DAG and apoptosis have been described in flies and mammals, suggesting that these phenotypes might reflect action on a common target. For example, in mammals accumulation of TAG in non-adipose tissues is associated with a form of cell death known as lipoapoptosis [19], whereas in *Drosophila*, increased ovarian cell death is associated with loss of an ovary-enriched form of acyl CoA:diacylglycerol transferase (DGAT), the enzyme that converts DAG into TAG [20]. Alternatively, the fat cell transcriptome is presumably different from that of retinal cells. Therefore, it is equally possible that *mir-14* targets different transcripts for distinct physiological purposes depending on the cell type and context in which it is expressed. Answers to these questions will come from identification and characterization of *mir-14* targets (discussed in the following section).

bantam promotes cell growth and inhibits apoptosis

The *bantam* locus was originally identified in a screen for genes that could promote tissue overgrowth when ectopically expressed [21]. Flies expressing the transcription factor GAL4 under the control of a tissue-specific promoter were crossed to flies carrying versions of the EP element. EP elements are engineered *P* elements that contain a GAL4-responsive promoter near one end. When this promoter is positioned appropriately (upstream of, and pointing into the 5′ end of a nearby gene) it can direct the expression of the nearby gene in the pattern in which GAL4 is expressed [22]. A cluster of EP elements located at locus 61C was associated with a GAL4-dependent increase in tissue size and cell proliferation. Conversely, disruption of this region with *P* element insertions or deletions gave rise to animals with defects indicative of decreased proliferation. As with *mir-14* above, the original characterization of the *bantam* locus did not point to any protein-coding genes as good candidates to encode bantam [21].

Brennecke and colleagues [8] used genomic rescue of the *bantam* loss-of-function phenotype to narrow the locus encoding bantam to ≈4 kb. A BLAST search of this sequence against the genome of the mosquito *Anopheles gambiae* identified a ≈90-nt region of significant similarity in which both sequences were predicted to fold into a hairpin structure reminiscent of a miRNA precursor. The results of northern blots, S1 nuclease mapping and overexpression of a small genomic fragment containing the predicted bantam precursor all confirmed that *bantam* in fact encodes an miRNA [8].

The induction of inappropriate proliferation through overexpression of genes such as *Drosophila* or mammalian E2F genes is often associated with increased apoptosis, a fact that limits the oncogenic potential of E2Fs [23]. By contrast, *bantam*-induced proliferation was not associated with an increase in apoptosis, and in fact *bantam* overexpression could suppress apoptosis induced by expression of E2F and its cofactor DP. These observations suggested that *bantam* also functioned as an apoptosis inhibitor [8]. The Hid transcript was identified as a likely target for this activity (although perhaps not the only target) based on several observations: (i) the Hid transcript 3′ untranslated region (UTR) contains potential *bantam* binding sites (defined in more detail below); (ii) *bantam* expression suppressed retinal cell death induced by Hid expression in the eye; and (iii) expression of a green fluorescent protein (GFP) reporter transgene with sequences from the Hid 3′ UTR was suppressed in the presence of *bantam*. Targets that mediate the ability of *bantam* to drive cell growth and proliferation remain to be identified.

Identifying miRNA targets and new miRNA regulators of cell death: the computational approach

The above observations with *mir-14* and *bantam* highlight the fact that to understand how miRNAs function to regulate cell death, proliferation, fat storage or any other process we must identify their mRNA targets. In plants this has proven to be reasonably straightforward because plant miRNAs typically show high levels of complementarity with their target transcripts, thus facilitating target identification through more standard homology searches [24]. By contrast, miRNAs in animals are in general not highly complementary to target transcripts. Therefore, other approaches must be used. Observations from several laboratories have begun to converge on a set of rules that define characteristics of functional miRNA–mRNA and siRNA–mRNA target pairs [11,12,25–27] (Figure 4). This work, in conjunction with computational approaches to genome-wide target prediction that utilize one or more of these rules, and often the assumption that physiologically relevant target sites will also be conserved in transcripts of homologous proteins from related species, has led to the generation of lists of candidate targets for many miRNAs in plants, flies and mammals [24,26,28–31].

The power of the computational approach to identifying miRNA death regulators is well illustrated by work in *Drosophila*. Enright [28], Stark [29] and colleagues carried out genome-wide searches for targets of *Drosophila melanogaster* miRNAs. Although their computational approaches and assumptions were somewhat different, both groups focused on the 3′ UTR as the site of likely targets. They each utilized steps that involve identification of local homologies, a calculation of miRNA–mRNA binding energy, and evolutionary conservation in *Drosophila pseudoobscura*. In addition, Stark *et al.* required a high level of homology between the second and the eighth nucleotide of the miRNA and its target. Complementarity within this region has been shown to be of particular importance in defining functional miRNAs (Figure 4). Both groups identified *hid* as a likely target of *bantam*, and as discussed above, Brennecke *et al.* [8] provided experimental support for this prediction. Both groups also identified members of the *mir-2* and *mir-13* families (which are identical for their first 11 base pairs) as likely regulators of the cell death activator *rpr*. Stark *et al.* also identified likely binding sites for the *mir-2* and *mir-13* family in the 3′ UTRs of two other cell death activators functionally related to *hid* and *rpr*, known as *grim* and *sickle*. As with *bantam* and *hid*, cell-based reporter assays confirmed that expression of *mir-2* does indeed lead to reduced expression of proteins encoded by transcripts that carry *rpr*, *grim* or *sickle* target sites [29]. These experiments demonstrate that, at least when overexpressed, *mir-2* and *mir-13* family members are likely to function as cell death inhibitors – that is, they probably can inhibit *rpr*-, *grim*- and *sickle*-dependent cell death (although this remains to be formally demonstrated). Whether these miRNAs normally function to regulate cell death *in vivo* depends on several factors. These include the relationship between the expression patterns and levels of these miRNAs and specific target mRNAs (*rpr*, *grim*, *sickle* and perhaps others that compete with them for miRNA binding). In addition, miRNA affinities for particular transcripts might well be influenced by the complement of RISC-associated RNA-binding proteins (Argonaute family members, Fragile X, Vasa Intronic Gene) present in the cell.

We have focused our discussion above on miRNA–mRNA target pairs predicted by Stark *et al.* and Enright *et al.* for which there is experimental support. However, many of the target predictions made by these two groups differ. This highlights several points. First, small changes in parameter values can lead to dramatic changes in the output. Second, these are first-generation target prediction programs whose predictive power will only increase as more genomes are sequenced, and as the importance of particular residues in miRNA–mRNA interactions is clarified through experiment. In summary, at this point in time computational approaches provide us with lists that have value as hypothesis generators. But these lists certainly contain false positives (genes that are predicted to be targets but are not physiologically significant) as well as false negatives (the lists fail to include some genes that are physiologically significant targets). This is because the factors that determine which miRNA–mRNA interactions occur, and the consequences of these interactions in any given cell type, are still being defined. Finally, it is important to note that our entire discussion has focused on roles for miRNAs as regulators of mRNA translation, as opposed to mRNA degradation or transcriptional silencing (Figure 1). This simply reflects the evolution of the field – the first miRNAs identified, *lin-4* and *let-7*, were shown to

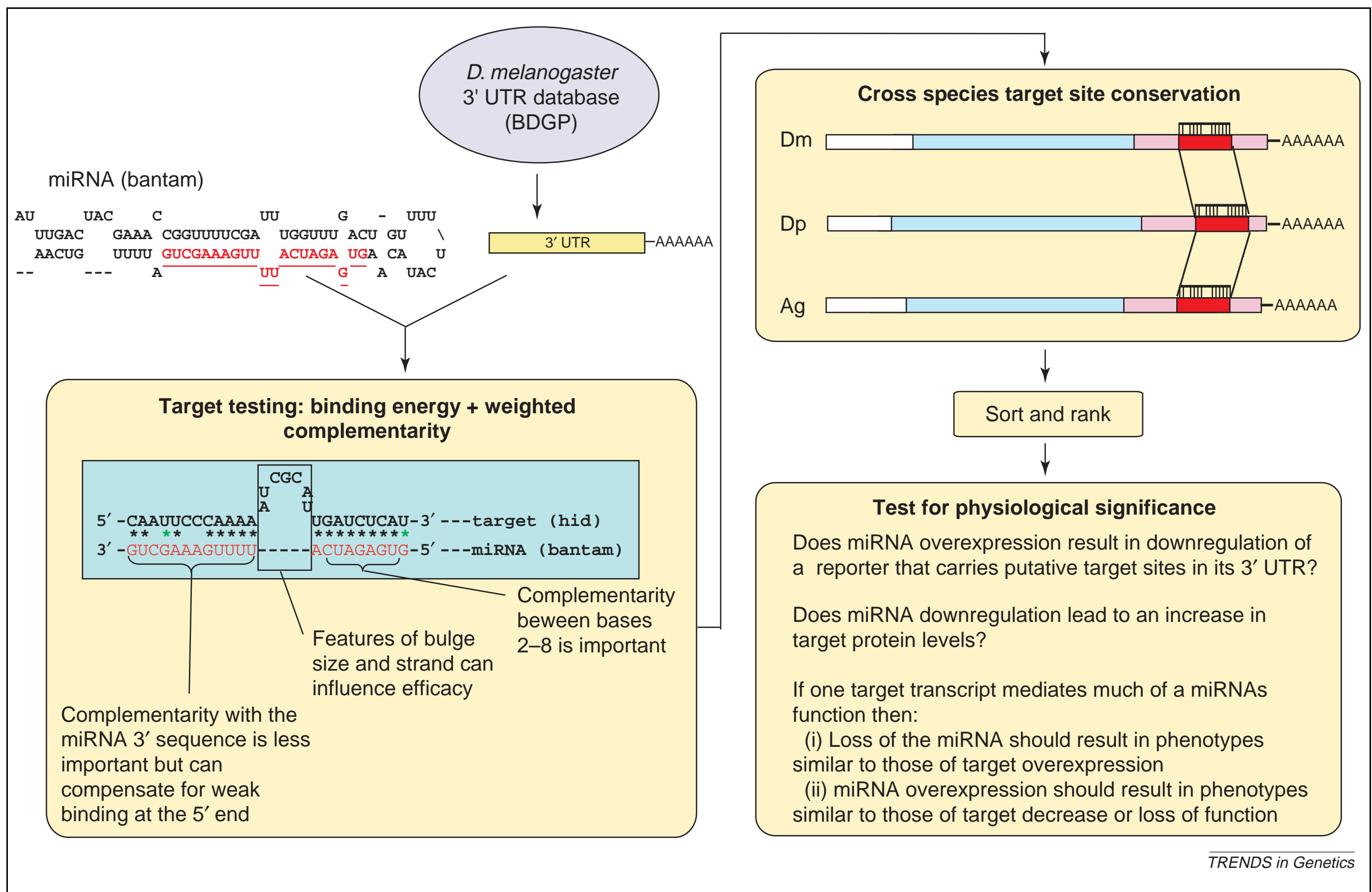

Figure 4. The computational approach to miRNA target identification. In a first step the mature microRNAs (miRNA) (in this case *bantam*, shown in red in the hairpin structure) is tested for binding energy and weighted complementarity against a database of *Drosophila* 3′ untranslated region (UTR) sequences. The use of a 3′ UTR database reflects the fact that known miRNA-binding sites are located in this region. However, the observation that binding sites within the coding region can also mediate translational suppression leaves it unclear whether this focus is warranted [53]. Binding energy is typically calculated using the RNA folding program mfold (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/). Complementarity weight matrices have been derived by identifying features shared by known productive miRNA–mRNA pairs [29] and through characterization of the activity of a large number of miRNA–mRNA mutants [26]. Some of the important features of productive miRNA–mRNA pairs identified by these approaches are indicated in the lower left panel. The presence of multiple binding sites and cooperativity between binding sites within an mRNA is also likely to be important for determining miRNA efficacy (not shown). Finally, for miRNAs that are conserved across species, it is likely that real target sites (their presence if not their exact location) will occur in transcripts encoding homologous proteins in related species (upper right panel). Algorithms such as those described above generate lists of candidates that must be tested for physiological significance. Some approaches are listed (lower right panel).

function as translational regulators. Given this, and the fact that in animals (but not plants) miRNAs do not show complete complementarity with other sites in the genome, most investigators have chosen to explore (with some notable successes) the hypothesis that newly identified miRNAs function through similar mechanisms. However, as noted above, RISC-bound small RNAs (siRNAs, and presumably also miRNAs) can induce significant levels of ‘off target’ cleavage of mRNAs to which they are not 100% complementary. In addition, siRNAs have very recently been shown to induce transcriptional gene silencing in human cells [32]. Therefore, it seems reasonable to imagine that some miRNAs will have different mechanisms of action.

Inactivating miRNAs

To determine the normal functions of specific miRNAs we need to be able to characterize their loss-of-function phenotypes (Figure 4). However, classical genetic mutations are available for less than a handful of miRNAs, and this situation is unlikely to change soon. Fortunately, it has recently been discovered that 2′-*O*-methyl oligoribonucleotides can be used to knock down, if not out, the activity of specific miRNAs. Briefly, when 2′-*O*-methyl oligoribonucleotides are introduced into cells in culture [33,34] or into animals directly (*C. elegans*) [33], they act as sequence-specific inhibitors of siRNA- or miRNA-bound RISC complexes to which they are complementary. This inhibition is stoichiometric, and irreversible [33]. Furthermore, biotinylated versions of these oligoribonucleotides can be used as affinity reagents to isolate and purify proteins associated with specific RNA-bound RISC complexes [33]. For both of these reasons 2′-*O*-methyl oligoribonucleotides are going to be very powerful tools to determine the functions of specific RISC-bound RNAs in different cell types, and the identities of protein cofactors that regulate these activities.

miRNA regulators of cell death in mammals?

miRNA regulators of cell death in *C. elegans* and mammals have not yet been identified. This might seem surprising, particularly in the case of *C. elegans*, because it was genetic screens in this organism that first demonstrated that cell death was under genetic control, and in

which several key components of the evolutionarily conserved cell death machine, namely Ced-4 (Apaf-1) and Ced-3 (caspases), were first described (reviewed in Ref. [35]). There are several probable explanations for this state of affairs. First, mature miRNAs are small (22 nt), making them difficult targets to hit in standard chemical- or radiation-based mutagenesis screens. By contrast, in *Drosophila*, cell death-inhibiting miRNAs were identified by virtue of phenotypes (loss-of-function, or gain-of-function in the case of overexpression) associated with nearby *P* element insertions. *P* elements in flies have a strong tendency to insert near the 5′ ends of genes [36,37], providing (in this case) a fortuitous mutagen (insertion site) bias that gives very small genes a target size comparable to that of larger protein-coding genes. Second, the initial screens for cell death regulators in *C. elegans* focused on identifying global regulators of cell death – mutants in which essentially all normally occurring cell deaths were blocked. miRNA cell death regulators might simply not play by these global roles, functioning instead to fine tune or modulate protein-based signals and effectors in specific cell types. In any case, given the generally high level of conservation of cell death signaling pathways between flies, worms and mammals [38] it is almost certain that miRNA regulators of cell death exist in organisms other than insects. In mammals, inhibition of cell death is an obligatory step on the path to cancer [23]. In addition, deregulation of translation and mutations in 3′ UTR sequences of protein-coding transcripts have been identified as important points of oncogenic regulation [39]. All of this suggests that tumors are likely to be a rich source from which to identify miRNA death regulators (see also the discussion in Ref. [40]). Transcriptional profiling of miRNAs up- or down-regulated in tumors provides one approach to identifying interesting candidates. For example, *mir-143* and *mir-145* were recently described as being downregulated in multiple stages of colorectal neoplasia [41], and *mir-17* is located in the last intron of the heterogeneous nuclear ribonucleoprotein K gene, which is overexpressed in multiple cancers [42]. Association of specific miRNAs with tumor-associated chromosomal aberrations provides a second approach, and several interesting candidates have recently been identified. *mir-15* and *mir-16* are deleted, or their expression is downregulated, in a majority of B-cell chronic lymphocytic leukemias [43]. A noncoding RNA known as B-cell receptor inducible gene (BIC), which encodes *mir-155*, was originally identified as a common retroviral integration site in avian leukosis virus [44]. BIC is upregulated in multiple tumor types, including avian leukosis, virus-induced lymphomas [45], Hodgkin's lymphoma [46], and pediatric Burkitt lymphoma [47]. Direct evidence that BIC upregulation is important for the development of these diseases comes from the finding that BIC cooperates with Myc in lymphomagenesis [48]. Finally, in a recent large-scale examination of 186 human miRNAs it was observed that several of these genes are located within regions of genomic DNA – albeit often defined rather broadly (<1 Mb) – found to be altered in specific cancers, for example, fragile sites or regions of DNA that are amplified or associated with loss of heterozygosity [49]. Altered expression was noted for several miRNAs. These observations do not prove that *mir-15, mir-16, mir-155* or any of these other miRNAs function by regulating cell death, because multiple alterations in cell physiology in addition to inhibition of death are required for successful tumor formation. Nonetheless, targets have been predicted for most of these [26,30], and the hypothesis is straightforward to test.

Cell death and miRNAs: the way forward

Multiple miRNAs that function as cell death inhibitors, *mir-14, bantam*, and probably members of the *mir-2* and/or *mir-13* family, have been identified in *Drosophila*. In addition, we identified three other death-suppressing miRNAs in our GMREP-based overexpression screen (P. Xu and B. Hay, unpublished). This screen of 8000 insertions is far from saturating because the *Drosophila* genome contains roughly 12 000 genes [50] and *P* elements show clear insertion-site specificity [51]. Therefore, it is very likely that more *Drosophila* miRNA death regulators remain to be identified. miRNA regulators of cell death in mammals have not yet been identified, but as discussed above, several interesting candidates await testing and more are certain to emerge as miRNAs are incorporated into transcriptional profiling experiments, and as target identification programs increase in predictive power. miRNAs are very easy to work with. Ectopic expression of the pre-miRNA (~70–80 nt) along with 100–200 bp of flanking sequence, under the control of Pol II promoters is, where tested, sufficient to bring about proper miRNA processing and function [7,8,52]. The results of ectopic expression experiments will tell us what processes specific miRNAs can affect. The use of 2′-*O*-methyl oligoribonucleotides to knock down miRNA function will tell us what processes these molecules normally regulate. The answers will arrive soon. Both natural and designed miRNAs are likely to be relatively invisible to the mammalian adaptive immune system because they do not encode proteins. If efficient methods can be developed for miRNA delivery, or the delivery of inactivating oligonucleotides, small RNAs have the potential to revolutionize our ability to intervene and modify cell physiology and behavior in the many human pathologies that involve deregulation of the cell death machine.

Acknowledgements

The authors' work described in this article was supported by The Ellison Medical Foundation, The Margaret E. Early Medical Trust, NIH grants GM070956 and GM57422 (B.A.H.) and a Gosney Fellowship (P.X.). We apologize to authors whose work could not be cited directly due to space limitations.

References

1 Vaux, D.L. and Flavell, R.A. (2000) Apoptosis genes and autoimmunity. *Curr. Opin. Immunol.* 12, 719–724
2 Fearnhead, H.O. (2004) Getting back on track, or what to do when apoptosis is de-railed: recoupling oncogenes to the apoptotic machinery. *Cancer Biol. Ther.* 3, 21–28
3 Mattson, M.P. (2000) Apoptosis in neurodegenerative disorders. *Nature Reviews Mol. Cell Biol.* 1, 120–129
4 Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297

5 Lai, E.C. (2003) microRNAs: runts of the genome assert themselves. *Curr. Biol.* 13, R925–R936

6 Nakahara, K. and Carthew, R.W. (2004) Expanding roles for miRNAs and siRNAs in cell regulation. *Curr. Opin. Cell Biol.* 16, 127–133

7 Xu, P. *et al*. (2003) The *Drosophila* microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. *Curr. Biol.* 13, 790–795

8 Brennecke, J. *et al*. (2003) *bantam* encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. *Cell* 113, 25–36

9 Ambros, V. (2001) microRNAs: tiny regulators with great potential. *Cell* 107, 823–826

10 Schramke, V. and Allshire, R. (2004) Those interfering little RNAs! Silencing and eliminating chromatin. *Curr. Opin. Genet. Dev.* 14, 174–180

11 Khvorova, A. *et al*. (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115, 209–216

12 Schwarz, D.S. *et al*. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115, 199–208

13 Degterev, A. *et al*. (2003) A decade of caspases. *Oncogene* 22, 8543–8567

14 Hay, B.A. (2000) Understanding IAP function and regulation: a view from *Drosophila*. *Cell Death Differ.* 7, 1045–1056

15 Hay, B.A. *et al*. (1995) *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* 83, 1253–1262

16 Hay, B.A. *et al*. (1997) P element insertion-dependent gene activation in the *Drosophila* eye. *Proc. Natl. Acad. Sci. U. S. A.* 94, 5195–5200

17 Vernooy, S.Y. *et al*. (2002) *Drosophila* Bruce can potently suppress Rpr- and Grim-dependent but not Hid-dependent cell death. *Curr. Biol.* 12, 1164–1168

18 Lagos-Quintana, M. *et al*. (2001) Identification of novel genes coding for small expressed RNAs. *Science* 294, 853–858

19 Unger, R.H. (2002) Lipotoxic diseases. *Annu. Rev. Med.* 53, 319–336

20 Buszczak, M. *et al*. (2002) Mutations in the *midway* gene disrupt a *Drosophila* acyl coenzyme A:diacylglycerol acyltransferase. *Genetics* 160, 1511–1518

21 Hipfner, D.R. *et al*. (2002) The *bantam* gene regulates *Drosophila* growth. *Genetics* 161, 1527–1537

22 Rorth, P. *et al*. (1998) Systematic gain-of-function genetics in *Drosophila*. *Development* 125, 1049–1057

23 Green, D.R. and Evan, G.I. (2002) A matter of life and death. *Cancer Cell* 1, 19–30

24 Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell* 14, 787–799

25 Doench, J.G. and Sharp, P.A. (2004) Specificity of microRNA target selection in translational repression. *Genes Dev.* 18, 504–511

26 Kiriakidou, M. *et al*. (2004) A combined computational-experimental approach predicts human microRNA targets. *Genes Dev.* 18, 1165–1178

27 Haley, B. and Zamore, P.D. (2004) Kinetic analysis of the RNAi enzyme complex. *Nat. Struct. Mol. Biol.* 11, 599–606

28 Enright, A.J. *et al*. (2003) MicroRNA targets in *Drosophila*. *Genome Biol.* 5, R1

29 Stark, A. *et al*. (2003) Identification of *Drosophila* microRNA targets. *PLoS Biol.* 1, 397–409

30 Lewis, B.P. *et al*. (2003) Prediction of mammalian microRNA targets. *Cell* 115, 787–798

31 Rajewsky, N. and Socci, N.D. (2004) Computational identification of microRNA targets. *Dev. Biol.* 267, 529–535

32 Morris, K.V. *et al*. (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* 305, 1289–1292

33 Hutvagner, G. *et al*. (2004) Sequence-specific inhibition of small RNA function. *PLoS Biol.* 2, 1–11

34 Meister, G. *et al*. (2004) Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* 10, 544–550

35 Metzstein, M.M. *et al*. (1998) Genetics of programmed cell death in *C. elegans*: past, present and future. *Trends Genet.* 14, 410–416

36 Spradling, A.C. *et al*. (1995) Gene disruptions using P transposable elements: an integral component of the *Drosophila* genome project. *Proc. Natl. Acad. Sci. U. S. A.* 92, 10824–10830

37 Bellen, H.J. *et al*. (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics* 167, 761–781

38 Vernooy, S.Y. *et al*. (2000) Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. *J. Cell Biol.* 150, F69–F76

39 Rajasekhar, V.K. and Holland, E.C. (2004) Postgenomic global analysis of translational control induced by oncogenic signaling. *Oncogene* 23, 3248–3264

40 McManus, M.T. (2003) MicroRNAs and cancer. *Semin. Cancer Biol.* 13, 253–258

41 Michael, M.Z. *et al*. (2003) Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol. Cancer Res.* 1, 882–891

42 Bomsztyk, K. *et al*. (2004) hnRNP K: one protein multiple processes. *BioEssays* 26, 629–638

43 Calin, G.A. *et al*. (2002) Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. U. S. A.* 99, 15524–15529

44 Clurman, B.E. and Hayward, W.S. (1989) Multiple proto-oncogene activations in avian leukosis virus-induced lymphomas: evidence for stage-specific events. *Mol. Cell. Biol.* 9, 2657–2664

45 Tam, W. *et al*. (1997) *bic*, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol. Cell. Biol.* 17, 1490–1502

46 van den Berg, A. *et al*. (2003) High expression of B-cell receptor inducible gene *BIC* in all subtypes of Hodgkin lymphoma. *Genes Chromosomes Cancer* 37, 20–28

47 Metzler, M. *et al*. (2004) High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. *Genes Chromosomes Cancer* 39, 167–169

48 Tam, W. *et al*. (2002) Avian *bic*, a gene isolated from a common retroviral site in avian leukosis virus-induced lymphomas that encodes a noncoding RNA, cooperates with c-*myc* in lymphomagenesis and erythroleukemogenesis. *J. Virol.* 76, 4275–4286

49 Calin, G.A. *et al*. (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl. Acad. Sci. U. S. A.* 101, 2999–3004

50 Rubin, G.M. *et al*. (2000) Comparative genomics of the eukaryotes. *Science* 287, 2204–2215

51 Liao, G.C. *et al*. (2000) Insertion site preferences of the P transposable element in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 97, 3347–3351

52 Chen, C.Z. *et al*. (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science* 303, 83–86

53 Saxena, S. *et al*. (2003) Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. *J. Biol. Chem.* 278, 44312–44319

54 Jackson, A.L. *et al*. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21, 635–637

55 Pal-Bhadra, M. *et al*. (2004) Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* 303, 669–672

56 Kuwabara, T. *et al*. (2004) A small modulatory dsRNA specifies the fate of adult neural stem cells. *Cell* 116, 779–793

57 Shi, Y. (2002) Mechanisms of caspase activation and inhibition during apoptosis. *Mol. Cell* 9, 459–470

58 Igaki, T. *et al*. (2002) Down-regulation of DIAP1 triggers a novel *Drosophila* cell death pathway mediated by Dark and DRONC. *J. Biol. Chem.* 277, 23103–23106

59 Muro, I. *et al*. (2002) The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J. Biol. Chem.* 277, 49644–49650

60 Rodriguez, A. *et al*. (2002) Unrestrained caspase-dependent cell death caused by loss of Diap1 function requires the *Drosophila* Apaf-1 homolog, Dark. *EMBO J.* 21, 2189–2197

61 Zimmermann, K.C. *et al*. (2002) The role of ARK in stress-induced apoptosis in *Drosophila* cells. *J. Cell Biol.* 156, 1077–1087