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# The *Drosophila* MicroRNA Mir-14 Suppresses Cell Death and Is Required for Normal Fat Metabolism

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## Summary

**MicroRNAs (miRNAs) are small regulatory RNAs that are between 21 and 25 nucleotides in length and repress gene function through interactions with target mRNAs [1, 2]. The genomes of metazoans encode on the order of several hundred miRNAs [3], but the processes they regulate have been defined for only two in *C. elegans* [4, 5]. We searched for new inhibitors of apoptotic cell death by testing existing collections of P element insertion lines for their ability to enhance a small-eye phenotype associated with eye-specific expression of the *Drosophila* cell death activator Reaper. Here we report the identification of the *Drosophila* miRNA mir-14 as a cell death suppressor. Loss of mir-14 enhances Reaper-dependent cell death, whereas ectopic expression suppresses cell death induced by multiple stimuli. Animals lacking mir-14 are viable. However, they are stress sensitive and have a reduced lifespan. Mir-14 mutants have elevated levels of the apoptotic effector caspase Drice, suggesting one potential site of action. Mir-14 also regulates fat metabolism. Deletion of mir-14 results in animals with increased levels of triacylglycerol and diacylglycerol, whereas increases in mir-14 copy number have the converse effect. We discuss possible relationships between these phenotypes.**

## Results and Discussion

We crossed flies that had small eyes due to the eye-specific expression of Reaper (Rpr) (GMR-Rpr flies) to publicly available lines with a lethal P element insertion on the second chromosome (Bloomington Stock Center) and examined transheterozygous progeny for enhancement or suppression of the GMR-Rpr small-eye phenotype. We identified one line, l(2)k10213 at 45F1, that was of particular interest (Figure 1A). Most l(2)k10213/GMR-Rpr transheterozygotes (87%, n = 300) died as pupae. However, all of those that survived to adulthood showed an enhanced GMR-Rpr small-eye phenotype (our unpublished data). These phenotypes persisted after the removal of a background lethal mutation on the l(2)k10213 chromosome. However, they were reverted after precise excision of the l(2)k10213 P element, indicating that they were due to the presence of this element. These observations suggested that the 45F1 region contained one or more genes that acted as suppressors of Rpr-dependent cell death in multiple contexts—in the eye and in other undefined tissues in which leaky expression of Rpr from the GMR promoter resulted in organismal death.

### Mir-14 Suppresses Cell Death

The annotated protein-coding genes nearest to the l(2)k10213 insertion are CG1888 and CG12931, 6.8 and 5.8 kb away, respectively (Figure 1A). However, the non-coding miRNA mir-14 [6] is located only 172 bp from the site of the l(2)k10213 insertion (Figure 1A). To explore the possibility that mir-14 functioned as a suppressor of Rpr-dependent cell death, we used imprecise P element excision to generate flies carrying a 533 bp deletion encompassing the mir-14 precursor (*mir-14*$\Delta^1$; Figure 1A). GMR-Rpr flies carrying one copy of this deletion showed an enhanced small-eye phenotype as well as a high-frequency lethality (Figure 1B). Both phenotypes were suppressed when *mir-14*$\Delta^1$/+;GMR-Rpr/+ heterozygotes carried a 3.4 kb fragment of genomic DNA encompassing the mir-14 region (mir-14$^{+3.4\,Kb}$) (Figures 1A and 1B), consistent with the hypothesis that these phenotypes were due to a loss of mir-14. We also increased the mir-14 copy number by introducing multiple copies of the mir-14$^{+3.4\,Kb}$ fragment into a GMR-Rpr background. As shown in Figure 1C, increasing the mir-14 dose to three or four copies led to further suppression of the Rpr-dependent small-eye phenotype.

To directly test the hypothesis that mir-14 alone was sufficient to act as a cell death suppressor, we generated flies that expressed a 118 bp fragment of genomic DNA containing the mir-14 precursor under GMR control (Figure 1A). The eyes of these flies (GMR-mir-14 flies) were wild-type in appearance (our unpublished data). As illustrated in Figure 2, GMR-mir-14 potently suppressed cell death induced by GMR-driven expression of Rpr, Hid, or Grim. Expression of mir-14 also suppressed late-onset retinal-cell death induced by expression of Dronc, an apical caspase that participates in much cell death signaling in the fly (reviewed in [7]). Importantly, however, GMR-mir-14 expression had little or no effect on the eye phenotypes induced by GMR-dependent expression of several other molecules, the long prodomain caspase Strica, whose mechanism of action and normal functions are unknown, or the Ras pathway negative regulator Tramtrack (see Supplemental Experimental Procedures, available with this article online, for details). Together, the results of these loss- and gain-of-function experiments argue that mir-14 is a dose-dependent suppressor of Rpr-, Hid-, Grim-, and Dronc-dependent cell death.

### Loss of Mir-14 Is Associated with Semilethality, Reduced Lifespan, and Stress Sensitivity

Mir-14 is expressed throughout *Drosophila* development and in the adult [8]. Loss of a cell death suppressor

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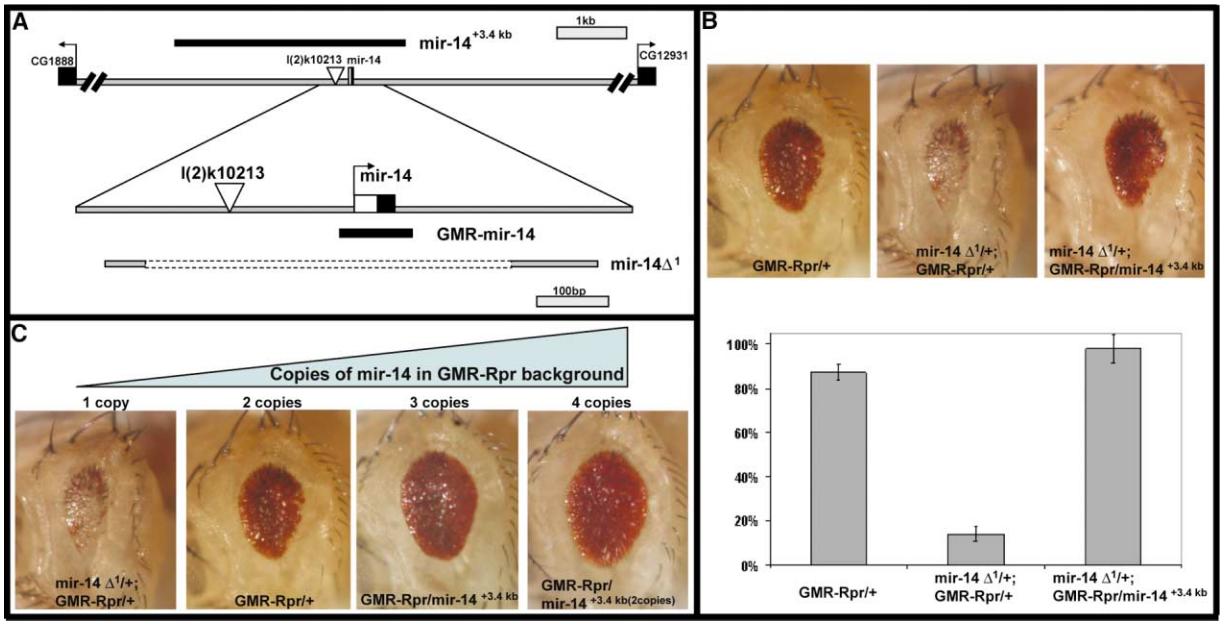


Figure 1. Mir-14 Acts as a Dose-Dependent Suppressor of Rpr-Dependent Cell Death

(A) Genomic organization of the mir-14 region. The mir-14 precursor is indicated by the white and black rectangle. The black region denotes sequences present in the mature mir-14 21 bp RNA. The direction of mir-14 transcription is indicated by the arrow. Locations of the l(2)k10213 P element, the *mir-14*$\Delta^1$ deletion, and the genomic sequences used to generate GMR-mir-14 and the mir-14 genomic rescue construct (mir-$14^{+3.4\ Kb}$) are indicated to scale.

(B) Upper panel: removal of one copy of mir-14 (*mir-14*$\Delta^1/+$) enhances GMR-Rpr-dependent cell death. This enhancement is suppressed in the presence of one copy of the mir-$14^{+3.4\ Kb}$ genomic rescue construct. All flies of the indicated genotypes had phenotypes similar to those shown. Lower panel: Removal of one copy of mir-14 enhances Rpr-dependent organismal lethality, and this is suppressed in the presence of one copy of the mir-$14^{+3.4\ Kb}$ genomic rescue construct. The indicated values are the means from ten independent experiments. The error bars indicate the standard deviation. N = 100 animals for each experiment.

(C) Mir-14 acts as a dose-dependent suppressor of GMR-Rpr-dependent cell death.

might be expected to result in reduced organismal viability and/or increased stress sensitivity. Homozygous *mir-14*$\Delta^1$ embryos from heterozygous parents hatched at a normal frequency (our unpublished data), and larvae survived to pupal stages at a rate similar to that of the wild-type (Figure 3A). However, most mir-14 larvae died

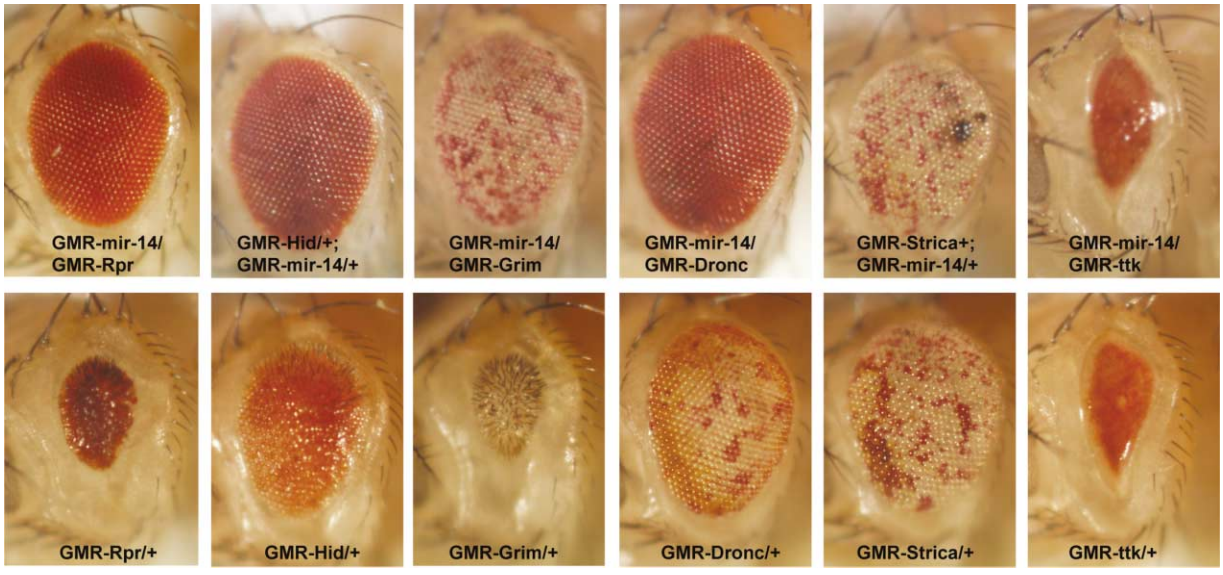


Figure 2. Expression of mir-14 Suppresses Retinal Cell Death Induced by GMR-Driven Expression of Rpr, Hid, Grim, or the Apical Caspase Dronc

However, mir-14 does not suppress death due to GMR-dependent expression of the caspase Strica or the Ras pathway negative regulator tramtrack (ttk).

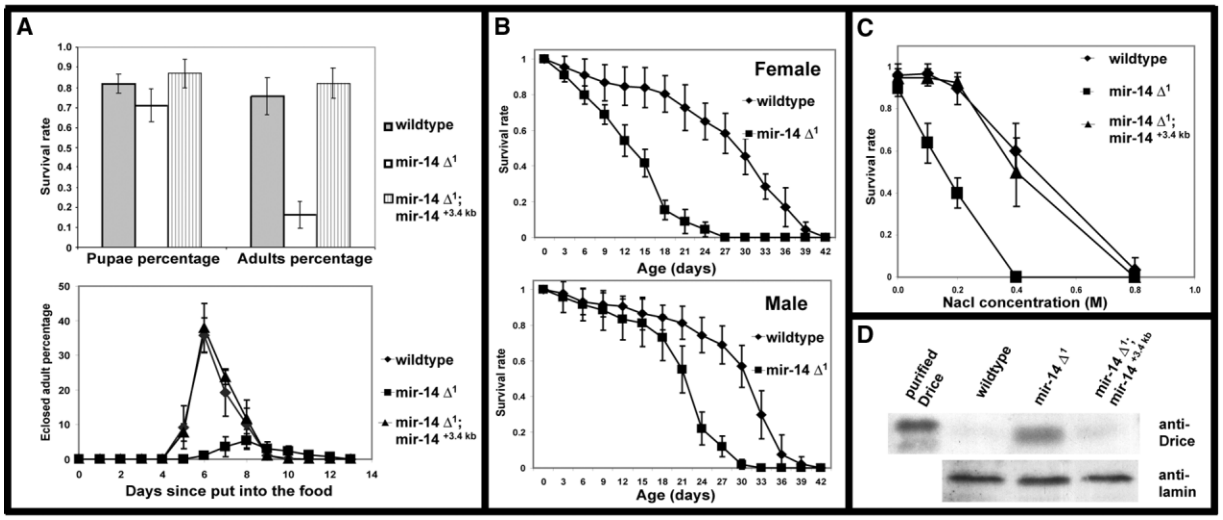


Figure 3. Loss of mir-14 Is Associated with Semilethality, Reduced Lifespan, Stress Sensitivity, and Increased Levels of the Caspase Drice

(A) Upper panel: *mir-14*$\Delta^1$ larvae pupate at a frequency similar to that of the wild-type (pupae percentage), but most die during pupal development (adult percentge). This lethality is rescued in the presence of two copies of the mir-14$^{+3.4\ Kb}$ genomic rescue fragment. Lower panel: Most *mir-14*$\Delta^1$ larvae do not eclose. Eclosion of those that do survive is moderately delayed with respect to the wild-type. Both phenotypes are rescued in the presence of two copies of the mir-14$^{+3.4\ Kb}$ genomic rescue fragment.

(B) *mir-14*$\Delta^1$ adults have a reduced lifespan compared to that of the wild-type (*w*−).

(C) *mir-14*$\Delta^1$ larvae are sensitive to salt stress (20 hr at the indicated concentration). Salt stress-induced lethality is rescued in the presence of two copies of the mir-14$^{+3.4\ Kb}$ genomic rescue fragment.

(D) Western blot of SDS-PAGE gel containing purified 6His-tagged Drice and extracts from adults of the indicated genotypes. Drice levels are elevated in *mir-14*$\Delta^1$ adults, and this is suppressed in the presence of two copies of the mir-14$^{+3.4\ Kb}$ genomic rescue fragment. The blot was reprobed with anti-lamin antibodies as a protein-loading control.

during pupal development (Figure 3A). Eclosion of those that survived was somewhat delayed with respect to the wild-type (Figure 3A). Both of these phenotypes were reverted when *mir-14*$\Delta^1$ homozygotes carried two copies of the mir-14$^{+3.4\ Kb}$ genomic fragment. *mir-14*$\Delta^1$ homozygous adults also had a decreased mean and maximal lifespan. This decrease was particularly marked in females (Figure 3B). Finally, homozygous *mir-14*$\Delta^1$ larvae were also much more susceptible than wild-type larvae to being killed by salt stress, an activator of the p38 mitogen-activated protein kinase (MAPK) pathway in *Drosophila* [9]. This phenotype was rescued by the presence of the mir-14$^{+3.4\ Kb}$ fragment (Figure 3C). In summary, flies lacking mir-14 showed compromised viability in multiple assays.

### Loss of Mir-14 Is Associated with Elevated Levels of the Apoptotic Effector Caspase Drice

The two miRNAs with known functions, lin-4 and let-7, are thought to regulate development by binding to the 3′ untranslated region of target transcripts and thereby repressing the translation of their products. In these examples, the analysis of genetic interactions provided important clues as to the identity of targets [1, 2]. In the absence of this sort of information, it is difficult to predict miRNA targets in animals. This is because base pairing between the mature miRNA and its target is imperfect and the rules that govern which base pair interactions are important are unknown. We searched for potential mir-14 binding sites in a number of apoptotic regulators, including Dronc, Rpr, Hid, and Grim (Figure S1). Potential target sites were identified in the transcripts of several genes, including Drice, Dcp-1, Scythe, SkpA, and Grim (however, the Grim target is present in the 3′UTR, which was absent in the GMR-Grim transgene) (Figure S1). Of these, Drice, an apoptotic effector caspase, was of particular interest. Drice is required for at least some cell deaths [10] and is activated by Dronc, which promotes cell death induced by Rpr, Hid, and Grim [11, 12]. We measured Drice levels in adults by using an anti-Drice antibody. Drice was elevated in *mir-14*$\Delta^1$ flies as compared to the wild-type, and this increase was suppressed in the presence of two copies of the mir-14-containing 3.4 kb genomic DNA fragment (Figure 3D). Whereas these observations alone do not prove that Drice is a direct target of mir-14, they do suggest that Drice is regulated, either directly or indirectly, by mir-14 levels.

### Mir-14 Regulates Fat Metabolism

We examined plastic sections from adult wild-type and *mir-14*$\Delta^1$ flies. The overall cellular architecture of heads from *mir-14*$\Delta^1$ flies was normal (Figure 4A). However, one striking phenotype was noted. Adipocyte lipid droplets were greatly enlarged in *mir-14*$\Delta^1$ flies (Figures 4A and 4B). This phenotype was suppressed in the presence of two copies of the mir-14-containing 3.4 kb genomic DNA fragment, consistent with the hypothesis that it was due to loss of mir-14 (Figure 4B).

Triacylglycerols (TAG) are the major component of adipocyte lipid droplets. All cells store small amounts of TAG that participate in phospholipid synthesis. However, adipocytes are the primary site of storage for an organism's overall needs. This suggested to us that *mir-*

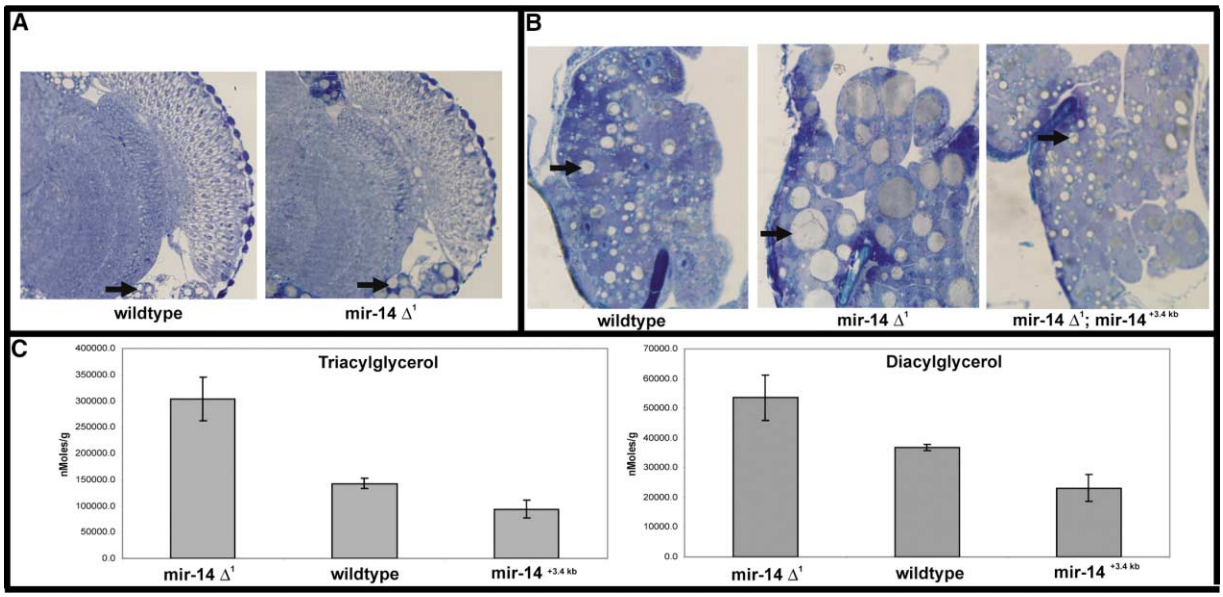


Figure 4. Mir-14 Regulates Fat Metabolism

(A) Plastic sections of wild-type and *mir-14*$\Delta^1$ adult heads. The retina is on the right, and the optic lobe is on the left. The overall cellular architecture of *mir-14*$\Delta^1$ adult heads is similar to that of the wild-type. However, lipid droplets (arrow) in adipocytes are greatly enlarged in *mir-14*$\Delta^1$ animals.

(B) Higher-magnification view of adult head fat bodies from three different genotypes. Lipid droplets in *mir-14*$\Delta^1$ adults are larger than those in the wild-type. This increase is suppressed in the presence of two copies of the genomic mir-14$^{+3.4\ Kb}$ rescue construct (*mir-14*$\Delta^1$;mir-14$^{+3.4\ Kb}$).

(C) Mir-14 is a dose-dependent regulator of triacylglycerol and diacylglycerol levels. Loss of mir-14 results in increased levels of total adult triacylglycerol and diacylglycerol. In contrast, increasing the mir-14 copy number to four through introduction of two copies of the mir-14$^{+3.4\ Kb}$ genomic fragment results in a decrease in triacylglycerol and diacylglycerol levels. N = 100 animals for each genotype. The indicated values are the means from three independent extractions and analyses. Error bars indicate the standard deviation.

*14*$\Delta^1$ adults might have elevated levels of TAG. In fact, the TAG content of *mir-14*$\Delta^1$ adults was increased about 2-fold over that of wild-type flies (Figure 4C). Dietary fats in *Drosophila* are transported from the midgut to their major storage site in the fat body as diacylglycerol (DAG) bound to a lipoprotein particle. At the fat body, DAG is converted into lipid droplet TAG for storage by the activity of an acyl CoA:diacylglycerol transferase (DGAT). Lipids are also mobilized from the fat body as DAG. TAG lipases produce DAG from TAG inside the adipocyte. DAG is then transported to the cell surface and added to a lipoprotein particle that is transported to target tissues through the hemolymph [13]. Diacylglycerol is also a precursor for the synthesis of multiple phospholipids and an important second messenger in multiple signal transduction pathways (reviewed in [14, 15]). Interestingly, diacylglycerol levels were also significantly elevated in *mir-14*$\Delta^1$ flies (Figure 4C). Flies that carried four copies of mir-14, two endogenous copies and two copies of the mir-14$^{+3.4\ Kb}$ fragment, had a set of phenotypes converse to the phenotype of animals lacking mir-14—a decrease in diacylglycerol and triacylglycerol levels (Figure 4C). Levels of several other lipid classes, including free fatty acids, cholesterol esters, lysophosphatidylcholine, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and total phospholipids were not significantly changed in flies lacking mir-14 or carrying additional copies of mir-14 (Figure S2). Together, these observations argue that mir-14 is a dose-dependent regulator of DAG and TAG metabolism in *Drosophila*.

## Concluding Remarks

We showed that mir-14 suppressed death induced by expression of Rpr, Hid, Grim, or the apical caspase Dronc. Furthermore, loss of mir-14 enhanced Rpr-dependent cell death, suggesting that mir-14 normally participates in death inhibition in some contexts. We have also identified, from gene activation screens [16], several other miRNAs that suppressed cell death when ectopically expressed in the fly eye (P.X., S.Y.V., and B.A.H., unpublished data). Together, these observations suggest that miRNAs are likely to constitute a heretofore hidden resource of cell death regulators. The identification of miRNAs that inhibit cell death is important for several reasons. It broadens the contexts in which miRNAs are known to function. In addition, it defines new points and mechanisms of cell death regulation. The identification of cell death-regulating miRNAs may also be important for understanding how cell survival is regulated in human disease. For example, it is likely that death-inhibiting miRNAs, being very small and noncoding, would not have been identified in previous screens for genes that promote oncogenesis by inhibiting cell death. They would also have been missed in experiments designed to identify candidate oncogenes through transcriptional profiling of normal and transformed cells because these experiments were not designed to detect miRNAs. Thus, it is reasonable to propose that deregulation of miRNA expression may contribute to the inappropriate survival that is so important for oncogenic progression.

Mir-14 dosage also regulates the levels of organismal DAG and TAG. DAG and TAG synthesis, storage, utilization, and degradation are regulated at many levels depending on cell type, as well as energy and signaling needs [17, 18]. Targets for mir-14 as a regulator of fat metabolism may be distinct from those that mediate its role as a cell death inhibitor. However, a number of described links between fat metabolism and apoptotic signaling suggest ways in which these phenotypes might be related. Overnutrition-induced obesity, lipodystrophy, and type II diabetes, as well as defects in TAG β-oxidation, lead to the accumulation of long-chain fatty acids in the form of TAG. TAG itself is probably not toxic. However, particularly in cells other than adipocytes, the surplus fatty acyl CoA can enter other nonoxidative pathways that promote cell dysfunction and/or cell death, including a form of caspase-dependent cell death known as lipoapoptosis (reviewed in [19]). Lipoapoptosis is driven, at least in part, by the de novo production of ceramide from excess fatty acyl CoA [20]. Rpr expression also promotes de novo production of ceramide [21, 22], and there is evidence that ceramide plays a role in mediating some of Rpr's pro-apoptotic effects [22]. It will be interesting to determine if mir-14 regulates the levels of fatty acyl CoA precursors or enzymes required for de novo ceramide synthesis. Also, as noted above, DAG is an important second messenger in multiple signal transduction pathways, some of which are linked to apoptosis induction [14, 15]. DAG-dependent signals are terminated by mechanisms that remove DAG. Mobilization of DAG into cellular TAG stores by DGAT is one quantitatively important pathway by which this is brought about [23]. *Drosophila* encodes multiple genes with homology to mammalian DGATs (CG31991, CG1941, and CG1942). Interestingly, mutations in the *Drosophila* gene *midway* (*mdy*, CG31991), which encodes a DGAT expressed predominantly in the female germline, lead to decreased nurse cell TAG levels and premature nurse cell death [24]. The mechanism by which *mdy* mutations promote nurse cell death is unknown. However, it is intriguing to speculate that loss of *mdy*, and perhaps mir-14 as well, leads to an increase in the levels of unesterified intracellular DAG and thereby promotes cell death signaling. An important question for the future will be whether regulation of mir-14 transcription or processing serves as a point of control for cell death, stress sensitivity, or fat storage in different environmental conditions or behavioral states.

### Supplemental Data

Supplemental Experimental Procedures as well as two supplemental figures are available with this article online at http://images.cellpress.com/supmat/supmatin.htm.

### Acknowledgments

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