RESEARCH COMMUNICATION

Drosophila miR-14 regulates insulin production and metabolism through its target, *sugarbabe*

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Energy homeostasis depends on insulin signaling in metazoans. Insulin levels reflect the nutritional status of the animal to control levels of circulating sugar and regulate storage of resources in the form of glycogen and fat. Over the past several years, evidence has begun to accumulate that insulin production and secretion, as well as cellular responsiveness to insulin, are subject to regulation by microRNAs. Here we present evidence that *miR-14* acts in the insulin-producing neurosecretory cells in the adult *Drosophila* brain to control metabolism. *miR-14* acts in these cells through its direct target, *sugarbabe*. *sugarbabe* encodes a predicted zinc finger protein that regulates insulin gene expression in the neurosecretory cells. Regulation of *sugarbabe* levels by nutrients and by *miR-14* combines to allow the fly to manage resource mobilization in a nutritionally variable environment.

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In mice, *miR-375* affects metabolism by regulating insulin secretion in pancreatic β cells (Poy et al. 2004). Mutant mice lacking *miR-375* show defects in glucose homeostasis, apparently due to reduced pancreatic β-cell mass (Poy et al. 2009). Mammalian microRNAs (miRNAs) also regulate insulin levels by other means. *miR-9* and *miR-96* have been implicated in insulin release through up-regulation of granuphilin, a negative regulator of secretion involved in vesicle docking (Lovis et al. 2008).

In *Drosophila*, miRNAs have been implicated in regulating insulin responsiveness in a metabolically important organ called the fat body. The fat body combines the functions of liver and adipose tissue and plays a central role in coordinating metabolism and growth of the organism during development. *miR-278* mutant flies show insulin resistance in most tissues, but most strongly in the fat body (Teleman et al. 2006). *miR-278* acts by regulating expression of the *expanded* gene. Expanded is best known as a membrane-associated FERM domain protein, which negatively regulates the Hippo signaling pathway (Cho et al. 2006; Hamaratoglu et al. 2006). However, the Hippo pathway does not appear to be involved in this context. A second miRNA, *miR-8*, also acts in fat body to control insulin signaling (Hyun et al. 2009). *miR-8* and its vertebrate ortholog, *miR-200*, indirectly activate phosphatidylinositol 3-kinase (PI3K) by repressing the expression of their conserved targets, U-shaped/FOG2. FOG2 protein binds to the regulatory subunit of PI3K and prevents formation of the active enzyme complex. Thus, loss of *mir-8* leads to reduced insulin sensitivity in the fat body, with effects on growth and metabolism.

In *Drosophila*, metabolic control by insulin production depends on insulin gene expression in a set of 14 neurosecretory cells in the brain (Brogiolo et al. 2001; Cao and Brown 2001; Ikeya et al. 2002). The developmental origin of these insulin-producing cells (IPCs) suggests an evolutionary relationship to mammalian pancreatic β cells (Wang et al. 2007; Clements et al. 2008). Flies lacking IPCs have elevated carbohydrate and fat levels (Rulifson et al. 2002; Broughton et al. 2005), indicating a role for IPCs in metabolic control. Previous reports have shown that *miR-14* mutant flies present defects related to apoptosis, stress response, survival, and metabolism (Xu et al. 2003). Misregulation of the Ecdysone receptor (EcR) was shown to be the cause of the pupal stage survival and metamorphosis defects and the reduced adult life span, but was not responsible for the obesity observed in the mutant adult flies (Varghese and Cohen 2007). In this study, we provide evidence that the metabolic function of *miR-14* depends on its activity in the IPCs, and explore its mechanism of action.

Results and Discussion

To explore the basis for the metabolic defect in *miR-14* mutant flies, we sought to use tissue-specific rescue of the mutant phenotype as a means to determine where miR-14 expression is required. We first confirmed that expression of a *UAS-miR-14* transgene under the control of a ubiquitously expressed Gal4-driver (*armadillo-Gal4*) could rescue the obesity phenotype. To assess obesity, we compared the ratio of total body triglyceride with total body protein. *miR-14* mutant flies showed an elevated fat to protein ratio, but this was restored to normal in the rescued mutant (Fig. 1A). Interestingly, the mutant was not rescued by expressing *UAS-miR-14* in the adipose tissue, using the fat body-specific driver *lsp2-Gal4* (Fig. 1A). This finding suggested that *miR-14* does not act in the adipose tissue to regulate triglyceride levels.

We made use of the observation that *miR-14* overexpression can make flies lean (Xu et al. 2003) to identify tissues in which miR-14 activity can influence fat levels (Supplemental Fig. S1). Expression of *miR-14* in the CNS using a pan-neuronal driver (Supplemental Fig. S1A)—or, more selectively, in the neurosecretory IPCs using *dILP2-Gal4*—produced lean flies (Fig. 1B). A *miR-14* lac-Z reporter transgene showed expression in the IPCs and in most other cells of the brain (IPCs are identified by *dilp-Gal4 UAS-nRFP*) (Fig. 1C; Supplemental Fig. S2A,B). Figure 1D shows expression of a miR-14 sensor (a ubiquitously expressed GFP reporter transgene with perfect miR-14 sites in the 3′ untranslated region [UTR]). Use of

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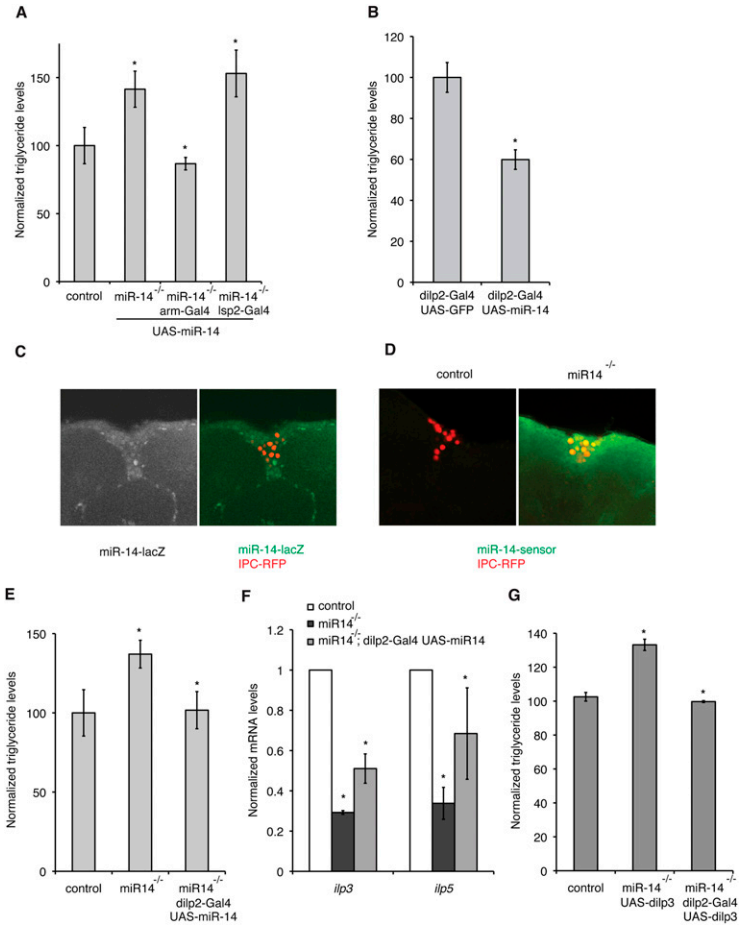

Figure 1. *miR-14* acts in the insulin-producing neurosecretory cells to control insulin production. (*A*,*B*,*E*,*G*) Histograms showing the ratio of total body triglyceride to total body protein (genotypes as indicated). Data are mean ± standard deviation (SD) based on three independent biological replicates. *P*-values were determined using Student's *t*-test. (*A*) Genetic rescue of *miR-14* mutants by expression of a *UAS-miR-14* transgene. $P = 0.02$ comparing control and *miR-14* mutant; $P = 0.01$ comparing *miR-14* mutant and *miR-14* mutant, *arm-Gal4-UAS-miR-14*; $P = 0.41$ comparing *miR-14* mutant and *miR-14* mutant, *lsp2-Gal4-UAS-miR-14*. (*B*) Overexpression of *miR-14* in the IPCs using *dILP2-Gal4*. $P = 0.03$ comparing *dilp2-Gal4 UAS-GFP* and *dilp2-Gal4 UAS-miR-14*. (*C*) *miR-14 lacZ* expression in the adult brain visualized using anti-β−Gal (green). IPCs labeled by *dILP2-Gal4*-directed expression of *UAS-nuclear RFP* (red). (*D*) *miR-14* sensor GFP in control and *miR-14* mutant adult brain (green). IPCs labeled by *dILP2-Gal4 UAS-nRFP* (red). (*E*) Rescue of the *miR-14* mutant by IPC-specific expression of *miR-14* using *dILP2-Gal4*. $P = 0.007$ comparing *miR14* mutant with *miR14* mutant, *dilp2-Gal4 UAS-miR-14*. (*F*) *Insulin-like peptide* mRNA levels measured by quantitative RT–PCR (qRT–PCR). Data represent three independent experiments, normalized to *RP49* mRNA and then to the level in control flies. For *ilp3*, $P = 2.4E\text{-}09$ comparing control with *miR-14* mutant; $P = 0.001$ comparing *miR14* mutant with *miR14* mutant, *dilp2-Gal4 UAS-miR-14*. For *ilp5*, $P = 1.7E\text{-}05$ comparing control with *miR-14* mutant; $P = 0.01$ comparing *miR14* mutant with *miR-14* mutant, *dilp2-Gal4 UAS-miR-14*. (*G*) Rescue of the *miR-14* mutant by IPC-specific expression of *UAS-dilp3* using *dILP2-Gal4*. *P*-value = 0.0001 comparing control and *miR-14* mutant, *UAS-ilp3* (without the Gal4 driver, *middle*); *P*-value = 3.3E-05 comparing *miR-14* mutant *UAS-ilp3* with *miR-14* mutant, *dilp2-Gal4 UAS-ilp3* (with the Gal4 driver).

perfect miR-14 sites allows for strong down-regulation of the sensor in *miR-14*-expressing cells. This is illustrated by comparing sensor levels in *miR-14* mutant clones and neighboring *miR-14*/+ heterozygous tissue in larval wing discs (Supplemental Fig. S2C). Similarly, *miR-14* sensor levels were very low in control brains, but increased considerably in *miR-14* mutant brains, indicating that miR-14 is active in the IPCs as well as other cells of the brain (Fig. 1D). Restoring *miR-14* expression selectively in the IPCs of otherwise *miR-14* mutant flies proved to be sufficient to restore fat levels to normal (Fig. 1E). Although *miR-14* is broadly expressed and active in the brain, this tissue-specific rescue identifies the IPCs as a key site of *miR-14* function in the control of metabolism.

How does *miR-14* expression in the IPCs affect fat levels? Previous work has shown that systemic insensitivity to insulin leads to an obese phenotype (Böhni et al. 1999; Tatar et al. 2001). Flies lacking IPCs or with reduced insulin-like peptide (ILP) levels store excess fat (Hwangbo et al. 2004; Broughton et al. 2005; Pospisilik et al. 2010). We therefore examined expression of the genes encoding IPC-specific ILPs in the *miR-14* mutant. The nutrient-sensitive *ilp* (*ilp3* and *ilp5*) (Ikeya et al. 2002) mRNA levels were reduced (Fig. 1F). This was partially rescued by *miR-14* expression in the mutant IPCs (Fig. 1F). The level of the non-nutrient-sensitive *ilp2* mRNA was also reduced, but to a lesser extent (Supplemental Fig. S1B). Interestingly, restoring *ilp3* expression in the IPCs by expression of a *UAS-ilp3* transgene was sufficient to restore normal fat levels in the *miR-14* mutant (Fig. 1G). This suggests that reduced *ilp* gene expression in the IPCs is sufficient to explain the metabolic effects of removing *miR-14*.

The observation that IPC-specific expression was sufficient to rescue the *miR-14* phenotype provided a means to search for *miR-14* targets that are functionally relevant in these cells. Expression profiling was performed using RNA isolated from adult heads of control flies, *miR-14* mutants, and IPC-rescued *miR-14* mutants. Of 810 mRNAs up-regulated by >1.5-fold ($P < 0.05$) in the mutant, 165 were reduced by >50% in the mutant with *miR-14* expression restored selectively in the IPCs. Forty-six of these had predicted *miR-14* target sites in their 3′UTRs. Ten of the 46 were among mRNAs enriched by Ago1 immunoprecipitation from S2 cells (Hong et al. 2009), where *miR-14* is abundantly expressed. These 10 mRNAs were selected for further analysis (Supplemental Table S1).

dILP2-Gal4 was used to express UAS-RNAi transgenes to reduce expression of the 10 candidates in the IPCs. This should mimic the effects of *miR-14* overexpression, which makes flies lean. Two of the candidates, *sugarbabe* and *fuzzy*, produced a lean phenotype when depleted by RNAi (Fig. 2A; Supplemental Table S1). As a second test, we asked whether overexpression in the IPCs could mimic the miRNA mutant phenotype. Overexpression of *fuzzy* had no effect, but *sugarbabe* overexpression in the IPCs led to elevated fat levels (Fig. 2B; Supplemental Table S1) and reduced levels of *ilp* transcripts (Fig. 2C; Supplemental Fig. S3A). Similar to what was observed in the *miR-14* mutant flies, the effect of *sugarbabe* overexpression was more pronounced on the levels of the two nutrient-sensitive *ilp* transcripts.

These experiments suggest that *sugarbabe* might be a biologically relevant target of miR-14 activity in the

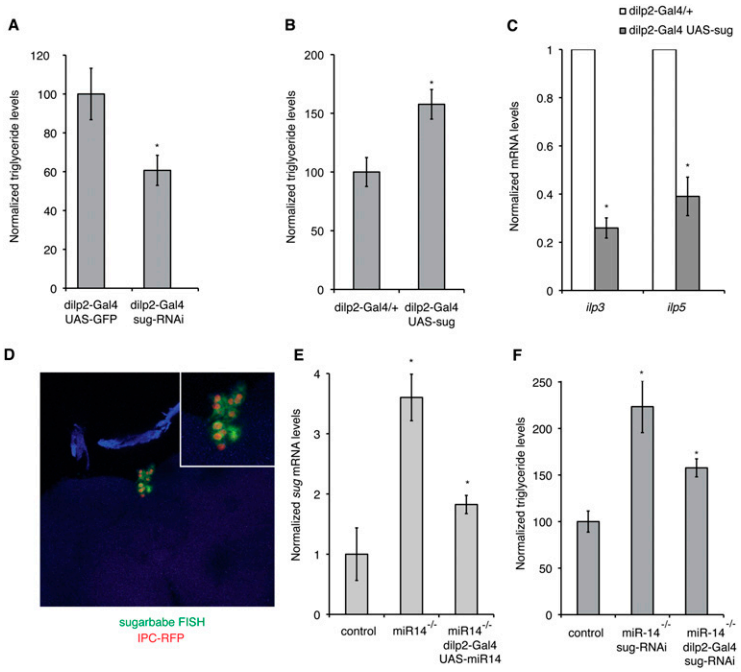

Figure 2. Genetic evidence that *miR-14* acts via *sugarbabe*. (*A*,*B*,*F*) Ratio of total body triglyceride to total body protein. (*C*,*E*) mRNA levels measured in flies of the indicated genotypes. Data are presented as mean ± SD based on three independent biological replicates. *P*-values determined using Student's *t*-test. (*A*) Depletion of *sugarbabe* by expression of a UAS-RNAi transgene in IPCs reduced fat levels. $P = 0.006$ comparing *dilp2-Gal4 UAS-GFP* with *dilp2-Gal4 UAS-sug-RNAi*. (*B*) Overexpression of *sugarbabe* in IPCs increased fat levels. $P = 0.002$ comparing *dilp2-Gal4*/+ with *dilp2-Gal4 UAS-sug*. (*C*) Overexpression of *sugarbabe* reduced expression of *ilp* mRNAs. $P = 1.2E-08$ for *ilp3* mRNA; $P = 2.8E-05$ for *ilp5* mRNA. (*D*) *sugarbabe* expression in the adult IPCs using FISH (green). IPCs marked by *dILP2-Gal4 nRFP* (red). (*E*) *sugarbabe* mRNA levels from expression-profiling data. $P = 0.0013$ comparing control with miR-14 mutant; $P = 0.0052$ comparing *miR-14* mutant with rescue-*miR-14* mutant, *dilp2-Gal4 UAS-miR-14*. (*F*) Partial suppression of *miR-14* mutant phenotype by IPC-specific depletion of *sugarbabe*. $P = 1.96E-05$ comparing control with *miR-14* mutant, *UAS-sug-RNAi* (without the Gal4 driver, *middle*); $P = 0.001$ comparing *miR-14* mutant, *UAS-sug-RNAi* with *miR-14* mutant, *dilp2-Gal4 UAS-sug-RNAi* (with the Gal4 driver).

IPCs. Fluorescent in situ hybridization (FISH) was used to visualize *sugarbabe* mRNA expression in the adult brain. *sugarbabe* mRNA was present in the IPC cells (identified by *dILP2-Gal4*-directed expression of a UAS-nRFP transgene) (Fig. 2D). Little or no *sugarbabe* mRNA was detected in other parts of the brain.

As a more stringent test of *sugarbabe* function, we asked whether limiting its overexpression selectively in the IPCs would reduce the severity of the *miR-14* mutant phenotype. IPC-specific expression of a *sugarbabe* UAS-RNAi transgene reduced *sugarbabe* mRNA levels in the adult head by ~30% and resulted in increased *ilp* transcript levels (Supplemental Fig. S3B). *sugarbabe* transcript levels increased approximately threefold in *miR-14* mutant head RNA (Fig. 2E; Supplemental Fig. S3C), and this was partially offset by IPC-specific expression of the UAS-RNAi transgene (Supplemental Fig. S3C). This also lowered fat levels (Fig. 2F). These assays point to *sugarbabe* as a functionally important *miR-14* target in the IPCs.

There is one predicted *miR-14* site in the *sugarbabe* 3′UTR (Fig. 3A). This site is conserved in the eight species of the *Drosophilia melanogaster* and *obscura* groups. To test its function in vivo, we prepared transgenic flies expressing a GFP reporter with the intact *sugarbabe* 3′UTR. GFP expression levels were higher in the IPCs and in other brain cells in *miR-14* mutant flies compared with wild-type controls (Fig. 3B). This indicates that endogenous levels of miR-14 are sufficient to negatively regulate gene expression via the *sugarbabe* 3′UTR in the IPCs in vivo. As a further test, we made use of a luciferase reporter with the intact *sugarbabe* 3′UTR and a second version in which the site was altered to disrupt pairing to the miRNA seed (Fig. 3A). Because S2 cells endogenously express *miR-14*, we asked whether depleting *miR-14* would lead to elevated expression of the luciferase reporters. The reporter with the mutated site was unchanged, but the reporter with the intact *miR-14* site showed a 40% increase in activity in *miR-14*-depleted cells (Fig. 3C). Reciprocally, overexpression of *miR-14* caused reduced luciferase activity from the intact reporter (Supplemental Fig. S3D). These results suggest that *miR-14* can act directly via the predicted target site to regulate *sugarbabe* levels.

In the laboratory, flies are reared on a nutritionally rich medium containing protein, amino acids, fats, and complex sugars. Natural environments offer a more variable nutrient supply, and the ability to adapt to changing conditions may confer an advantage. Like other animals, flies store energy in the form of glycogen and fat. We examined the contribution of *miR-14* and *sugarbabe* to their response to nutrient stress. For these experiments, larvae were reared under controlled growth conditions on rich medium, and adult flies were aged on this medium for 4 d before being deprived of nutrients. *miR-14* mutant flies were more sensitive to nutrient deprivation, as were flies selectively overexpressing *sugarbabe* in the IPCs, which phenocopies the *miR-14* mutant (Fig. 4A,B). We confirmed that the reduced survival of the *miR-14* mutants under these conditions was due to elevated *sugarbabe* levels by selectively expressing the *sugarbabe* RNAi transgene in the IPC cells of the *miR-14* mutant. Partially compensating for the elevated *sugarbabe* mRNA level improved survival of the mutant flies (Fig. 4C)

At the start of the experiment, before nutrient deprivation, fat levels were higher in the mutant (Fig. 4D), but glycogen levels were lower—an expected outcome of reduced insulin signaling (Fig. 4E). During nutrient deprivation, glycogen drops more quickly than in control flies, but reaches similar low levels by 36 h. Fat reserves were initially mobilized more slowly, but then dropped sharply at the stage when the mutant flies begin to die (Fig. 4D). Thus, there is an initial metabolic imbalance in the *miR-14* mutants, with nutrient reserves shifted more toward fat storage, perhaps at the expense of glycogen. The resulting imbalance in nutrient mobilization may be responsible for the impaired survival of the mutant flies when nutrient-deprived.

Overexpression of *sugarbabe* in the IPCs is sufficient to mimic the effects of the *miR-14* mutant on fat storage, expression of the nutritionally sensitive *ilp* transcripts, and starvation sensitivity. To further explore their function in this context, we asked whether their expression in

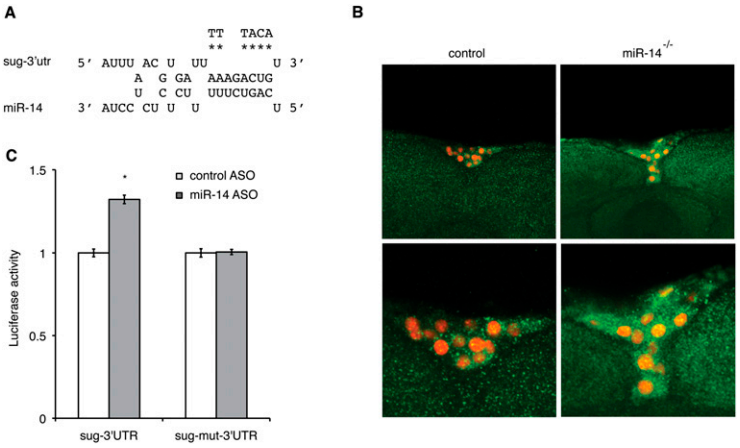

Figure 3. *miR-14* regulates *sugarbabe* expression. (*A*) Predicted *miR-14* target site in the *sugarbabe* 3′UTR. Asterisks indicate residues changed in the target site mutant. (*B*) *sugarbabe* 3′UTR reporter transgene showing GFP expression in control and *miR-14* mutant brains (green). IPCs marked by *dILP2-Gal4 nRFP* (red). (*C*) Luciferase assays showing regulation of a *sugarbabe* 3′UTR reporter. S2 cells were transfected to express a firefly luciferase *sugarbabe* 3′UTR reporter or a version of the reporter with the site mutated. Cells were cotransfected with a renilla luciferase reporter as a control for transfection efficiency. Data show the ratio of firefly to Renilla luciferase activity. Cells were treated with antisense oligonucleotide to deplete *miR-14* or with a control oligonucleotide. Data represent mean ± SD for three independent biological replicates. P = 4.9E-05 (Student's t-test).

the adult head was nutrient-responsive. *sugarbabe* has been shown previously to be up-regulated in the gut, malpighian tubules, and fat body tissues of larvae fed on a high-sugar diet (Zinke et al. 2002). We found that *sugarbabe* mRNA levels decreased by >50% in RNA from adult heads (Fig. 5A), whereas *miR-14* levels were not affected significantly (Fig. 5B). The change in *sugarbabe* levels was also seen in nutrient-deprived *miR-14* mutants (Fig. 5C), showing a miR-14-independent change of *sugarbabe* during starvation. Thus, *sugarbabe* expression is controlled in the IPCs by two independent means.

Sugarbabe encodes a predicted zinc finger protein (Zinke et al. 2002). Our findings suggest that *sugarbabe* levels in the IPC are under nutritional regulation, and that this in turn controls the levels of *ilp* mRNAs. Sugarbabe might act as a transcription factor to regulate *ilp* gene expression, but we do not exclude the possibility that its effects are post-transcriptional, affecting RNA processing or stability.

How do the nutrition-dependent changes in *sugarbabe* expression correlate with regulation of *ilp* transcript levels? Based on the data presented so far, the observed reduction in *sugarbabe* levels upon nutrient deprivation is expected to elevate *ilp* transcript levels. Yet, *ilp* levels decrease under these conditions. To address this issue, we examined the effects of offsetting the decrease in *sugarbabe* levels by expressing *UAS- sugarbabe* in the IPCs under *dilp2-Gal4* control. Under these conditions, the levels of *ilp3* and *ilp5* transcripts decreased by considerably more than in the nutrient-deprived controls (Fig. 5D). This indicates that down-regulation of *sugarbabe* during nutrient deprivation serves to limit the effects of nutrient deprivation on *ilp* gene expression.

Our findings highlight the importance of *sugarbabe* levels in the IPCs as a regulator of energy balance. Under normal circumstances, elevated fat levels can confer resistance to starvation (Hader et al. 2003; Gronke et al. 2007). However, flies with defects in fat mobilization can be hypersensitive to starvation despite being obese (Gronke et al. 2007). Impairing insulin signaling has been shown to enhance fat storage (Böhni et al. 1999; Tatar et al. 2001; Hwangbo et al. 2004; Broughton et al. 2005), but it can also sensitize flies to starvation in some contexts (Clancy et al. 2001). *miR-14* mutants show a similar syndrome of low insulin and obesity coupled with starvation sensitivity. Our findings suggest that nutritional regulation of *sugarbabe* might serve to limit the reduction of insulin expression during starvation. We speculate that maintaining a sufficient level of insulin activity might be important to allow mobilization of nutrient reserves from fat stores. The finding that *sugarbabe* is nutritionally controlled, while *miR-14* levels are not, provides the opportunity for an interplay between nutritionally dependent and independent regulation of insulin production.

Materials and methods

Reagents and fly strains

Reagents and fly strains used were as follows: *miR-14* mutant and *P{lacW}miR-14^{k10213}* (Xu et al. 2003); *UAS-miR-14* and *tub-miR-14* (Varghese and Cohen 2007); *UAS-sugarbabe* (Zinke et al. 2002); *dilp2-Gal4* and *UAS-dilp3* (Ikeya et al. 2002). *armadillo-Gal4*, *elav-Gal4*, and *UAS-GFP* were from the Bloomington Stock Center (http://flybase.bio.indiana.edu). *lsp2-Gal4* was provided by Bassem Hassan. *UAS-sugarbabe-RNAi* (Dietzl et al. 2007). Other RNAi lines were from Vienna *Drosophila* RNAi Center (VDRC), National Institute of Genetics (NIG) RNAi Stock Center, and Bloomington Stock Center. miR-14-sensor GFP was generated as described (Brennecke et al. 2003). sug-3′UTR GFP and luciferase reporters were generated as described (Varghese and Cohen 2007).

Conditions for phenotype analysis

Controlled growth conditions were used for all experiments related to metabolism. First instar larvae were collected within 2–3 h of hatching. GFP balancers were used to allow genotyping. Fifty larvae were placed in fresh vials to maintain uncrowded conditions. Adult males were picked within 6 h of emergence and aged for 5 d (10 per vial) before triglyceride measurement or quantitative real-time PCR assays. For starvation, flies were collected from similar conditions. They were then transferred to normal fly food vials or vials containing only 1.5% agar and collected at the indicated time points.

Triglyceride and glycogen measurements

Batches of 10 flies were homogenized using Sartorius Potter-S tissue homogenizer. After 5 min of heat inactivation at 70°C and centrifugation at 13000 rpm for 3 min, triglyceride and protein levels were measured using the Sigma Triglyceride kit and Bio-Rad protein assay reagent. Colorimetric analysis was performed using a TECAN microplate reader in 96-well format. Sample preparation for glycogen measurement was similar to triglycerides, following the manufacturer's protocol (BioVision). Data were normalized to total protein.

Quantitative PCR (qPCR)

Batches of 20 flies were collected, snap-frozen in liquid nitrogen, and shaken to separate head and body. Total RNA was extracted from heads using Trizol. RNA samples were treated with DNase1 (Qiagen RNeazy

kit). Reverse transcription used oligo-dT primers and SuperScript RT-III (Invitrogen) and the cDNAs used for qPCR with Power SYBR Green (Applied Biosystems) and ABI-7500 Fast qPCR machine.

The following primers were used: *dilp2* FP, 5′-GGCCAGCTCCACAG TGAAGT-3′; *dilp2* RP, 5′-TCGCTGTCGGCACCGGGCAT-3′; *dilp3* FP, 5′-CCAGGCCACCATGAAGTTGT-3′; *dilp3* RP, 5′-TTGAAGTTCACG GGGTCCAA-3′; *dilp5* FP, 5′-TCCGCCCAGGCCGCAAACTC-3′; *dilp5* RP, 5′-TAATCGAATAGGCCCAAGGT-3′; *sugarbabe* FP, 5′-CCGCCA GCGATTTCGTATG-3′; *sugarbabe* RP, 5′-GCCAGTGCATCCAAGGT GTC-3′.

Transfection and antisense oligonucleotide-mediated depletion of miR-14 in S2 cells

S2 cells were transfected in 24-well plates using 250 ng of *tubulin-miR14* plasmid DNA or the empty vector control, 25 ng of firefly luciferase DNA or sug-3′UTR or mutant-3′UTR luciferase reporter DNA, and 25 ng of Renilla luciferase DNA as a transfection control. Dual-luciferase assays were performed 60 h post-transfection according to the manufacturer's protocol (Promega). Antisense oligos were designed and S2 cells were transfected according to Horwich and Zamore (2008): *miR-14* ASO, 5′-AGA GAUAGGAGAGAGAAAAAGACUGAAGGAU-3′; control ASO (miR-14 shuffle), 5′-AGAGAUAAGUAGGAGUGAGAAGAGAAAGGAU-3′. ASOs incorporated 2′-O-methyl-modified nucleotides and had a 3′-cholesterol modification (Dharmacon). ASOs and luciferase reporter constructs were transfected using Dharmafect 4. Cells were harvested 72 h later, and dual-luciferase assays were performed.

Microarray analysis

Microarray profiling was performed by the EMBL Gene Core using Affymetrix *Drosophila* 2.0 arrays. RNA was amplified and labeled using standard protocols for hybridization, washing, and data acquisition (Fluidics Station 400, GeneArray 2500 scanner, Affymetrix Microarray suite version 5.1). Data were normalized using CARMAWeb version 1.4.

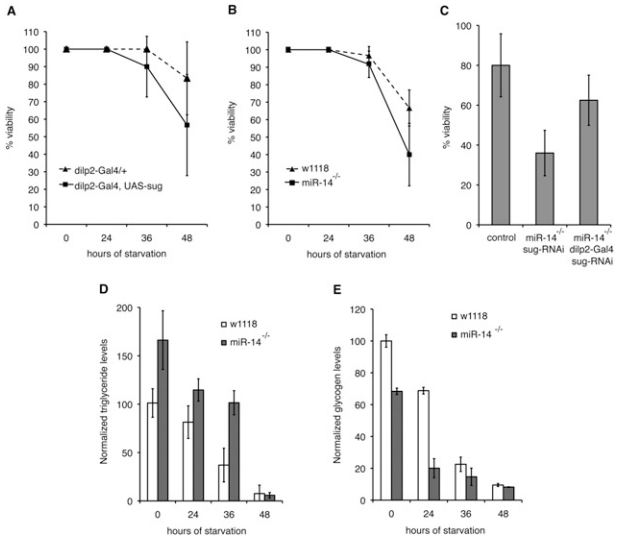

Figure 4. *miR-14* effects on nutrient deprivation. (*A*,*B*) Survival curves of adult male flies during nutrient deprivation. Flies were reared and aged under controlled conditions and deprived of nutrients at $t = 0$. Data represent three independent biological replicates. (*A*) $P = 0.105$ (36 h) and $P = 0.005$ (48 h) comparing *dilp-Gal4* with *dilp-Gal4 UAS-sug*. (*B*) $P = 0.045$ (36 h) and $P = 0.13$ (48 h) comparing control w^{1118} with *miR-14* mutant. (*C*) Partial suppression of the reduced viability of *miR-14* mutants by IPC-specific depletion of *sugarbabe* at 48 h. $P = 0.0005$ control versus *miR-14, UAS-sug-RNAi* (without the Gal4 driver, *middle*); $P = 0.001$ comparing *miR-14* mutant, *UAS-sug-RNAi* with *miR-14* mutant, *dilp2-Gal4 UAS-sug-RNAi* (with the Gal4 driver). (*D*) Ratio of triglyceride to protein on nutrient-deprived flies, reared as in *B*. $P = 0.02$ (0 h), $P = 0.02$ (24 h), $P = 0.003$ (36 h), and $P = 0.38$ (48 h) comparing control w^{1118} with *miR-14* mutant. (*E*) Glycogen levels of nutrient-deprived flies, reared as in *B*. $P = 0.001$ (0 h), $P = 9.28\text{E-}05$ (24 h), $P = 0.06$ (36 h), and $P = 0.04$ (48 h) comparing control w^{1118} with *miR-14* mutant.

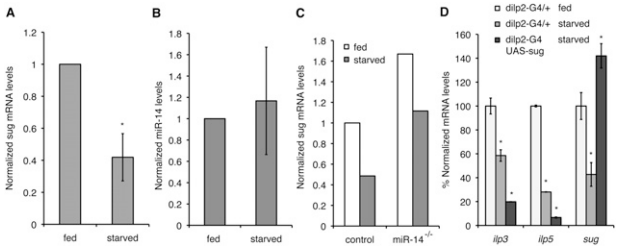

Figure 5. Responses to nutrient deprivation. (*A*) *sugarbabe* mRNA levels measured by qRT–PCR in w^{1118} control flies reared under controlled conditions. "Starved" indicates flies deprived of nutrients for 24 h. Data represent three independent biological replicates. $P = 0.0002$. (*B*) *miR-14* miRNA levels measured by qPCR. $P = 0.3$. (*C*) *sugarbabe* mRNA levels compared in control and *miR-14* mutants under fed and nutrient-deprived conditions. (*D*) *ilp3*, *ilp5*, and *sugarbabe* mRNA levels measured by qRT–PCR in *dilp2-Gal4/+* control flies and *dilp2-Gal4 UAS-sugarbabe* (Gal4-directed *sugarbabe* expression in IPCs) reared and treated as in *A*. Data represent three independent biological replicates. For *ilp3* levels, $P = 0.018$ comparing fed versus starved control flies; $P = 0.007$ comparing starved control and *sugarbabe*-overexpressing flies. For *ilp5* levels, $P = 0.003$ comparing fed and starved control flies; $P = 0.0002$ comparing starved control and *sugarbabe* overexpression flies. For *sugarbabe* levels, $P = 0.03$ comparing fed and starved control flies; $P = 0.01$ comparing starved control and *sugarbabe*-overexpressing flies.

FISH and antibody labeling

The *sugarbabe* antisense RNA probe was synthesized using the DIG-labeling kit (Roche). Anti-DIG-POD primary antibody was used and detected using the Tyramide Signal Amplification kit from Perkin Elmer following standard protocols. Rabbit anti-β-galactosidase antibody was from Cappel. Mouse anti-GFP antibody was from Molecular Probes.

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