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PATTERNS & PHENOTYPES

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The microRNA *miR-33* is a pleiotropic regulator of metabolic and developmental processes in *Drosophila melanogaster*

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Abstract

Background: *miR-33* family members are well characterized regulators of cellular lipid levels in mammals. Previous studies have shown that overexpression of *miR-33* in *Drosophila melanogaster* leads to elevated triacylglycerol (TAG) levels in certain contexts. Although loss of *miR-33* in flies causes subtle defects in larval and adult ovaries, the effects of *miR-33* deficiency on lipid metabolism and other phenotypes impacted by metabolic state have not yet been characterized.

Results: We found that loss of *miR-33* predisposes flies to elevated TAG levels, and we identified genes involved in TAG synthesis as direct targets of *miR-33*, including *atpcl*, *midway*, and *Akt1*. *miR-33* mutants survived longer upon starvation but showed greater sensitivity to an oxidative stressor. We also found evidence that *miR-33* is a negative regulator of cuticle pigmentation and that *miR-33* mutants show a reduction in interfollicular stalk cells during oogenesis.

Conclusion: Our data suggest that *miR-33* is a conserved regulator of lipid homeostasis, and its targets are involved in both degradation and synthesis of fatty acids and TAG. The constellation of phenotypes involving tissues that are highly sensitive to metabolic state suggests that *miR-33* serves to prevent extreme fluctuations in metabolically sensitive tissues.

KEYWORDS

lipid homeostasis, oogenesis, pigmentation, triacylglycerol

1 | INTRODUCTION

Metabolic processes are highly regulated, and metabolic dysregulation underlies many common diseases, such as cancer, obesity, and cardiovascular disease. A better understanding of the importance of non-coding RNAs such as microRNAs (miRNAs) in regulating metabolism has emerged over the last decade.[1] Genes encoding microRNAs are transcribed and processed into short (~22 nt) mature miRNAs that bind to multiple mRNA targets through base-pairing and generally inhibit gene expression through decreased stability or inhibition of translation of those targets.[2] Although many miRNA genes are located in intergenic regions, some are actually

located within the introns of other genes and are co-transcribed along with their host gene, raising the interesting possibility of an interplay between the host gene and its resident miRNA in regulating similar processes. The *miR-33* family of miRNAs is one such example. In humans, *miR-33a* and *miR-33b* are located within the introns of the *SREBP2* and *SREBP1* genes, respectively. The sterol regulatory element binding protein (SREBP) transcription factors play a major role in driving expression of genes implicated in fatty acid and sterol synthesis in mammals.[3,4] Of note, these proteins are first produced as inactive forms in the endoplasmic reticulum. Changes in lipid concentrations lead to proteolytic release of the active transcription factors that coordinate a response to maintain cellular cholesterol and fatty acid homeostasis.[5,6]

Several studies have demonstrated that *miR-33a* cooperates with SREBP2 to maintain cellular lipid levels by reducing cellular cholesterol export and reducing fatty acid degradation in mammals.[7-11] Specifically, *miR-33a* reduces cellular cholesterol transport through its targeting of the transcript coding for the ABCA1 transporter. Furthermore, we and others have demonstrated that *miR-33* members regulate fatty acid catabolism by targeting transcripts coding for three main components of fatty acid β-oxidation (CPT1A, HADHB, CROT).[7,12] *miR-33* can also target mRNAs involved in insulin signaling and mitochondrial function, including *IRS2*, *AMPK*, and *PGC1alpha*.[12-14] In support of *miR-33* playing a role in regulating metabolism in mammals, studies have reported that *miR-33a* knockout mice have higher total and HDL cholesterol levels and are more susceptible to developing obesity, insulin resistance, and liver steatosis especially when fed a high fat diet.[8,15,16]

The *miR-33* family is highly conserved during evolution. In *Drosophila melanogaster*, *miR-33* is located in an intron of the single *SREBP* homolog. Notably, *D melanogaster* are cholesterol auxotrophs, and SREBP is activated by reduced levels of palmitate rather than changes in cholesterol levels as in the case of mammalian SREBP2.[17,18] In turn, SREBP target genes in flies are mainly involved in fatty acid and phospholipid synthesis but not in cholesterol synthesis.[18,19] Consistent with the hypothesis that *miR-33* regulation of metabolism is also conserved in flies, we previously showed that the *Drosophila* ortholog of *Cpt1* (*whd*) is also targeted by *miR-33* in flies.[7] In accordance with this finding, overexpression of *miR-33* in larval fat body partially prevents the reduction of triacylglycerol (TAG) levels during starvation, and ubiquitous overexpression throughout development causes elevated TAG levels in adults.[12,20] Similar to *miR-33* mutant mice, loss of *miR-33* in flies has been reported to cause no gross developmental defects, but defects in both larval and adult ovaries have been reported.[21,22] However, these studies analyzing loss of *miR-33* in flies did not report on other phenotypes related to metabolism such as TAG levels, responses to diet and stressors, or cuticle pigmentation.

In this present study, we aimed to further characterize the role of *miR-33* in various metabolic and developmental processes in *D melanogaster*. We found that flies lacking *miR-33* show various signs of metabolic dysfunction involving multiple tissues and that *miR-33* can target multiple genes involved in TAG synthesis. This indicates that *miR*-33 plays a conserved role in regulating systemic metabolic homeostasis and related developmental processes.

2 | RESULTS

2.1 | Flies lacking *miR-33* are predisposed to having elevated TAG levels

To characterize the role of *miR-33* in *D melanogaster*, we used CRISPR/Cas9 to introduce a small deletion comprising *miR-33*. We designed gRNAs to target areas flanking the genomic region that gives rise to the mature *miR-33-5p* transcript (Figure 1A). After injecting the gRNA constructs into embryos expressing Cas9 in the germline, progeny were screened for deletions of this region that were randomly produced through non-homologous end joining repair of the breaks.[23] The resulting $miR\text{-}33^{F11}$ allele has a 53 bp deletion within the *SREBP* intron that deletes the mature *miR-33-5p* sequence and some of the upstream pre-miRNA sequence (Figure 1A). We used this allele in conjunction with a previously reported null allele that was generated, in parallel to our own, in another lab. This other allele, designated as $miR\text{-}33^{KO}$, contains a larger 134 bp deletion that includes the entire pre-miRNA and a short region upstream.[21] We backcrossed these two mutant lines to a w^{1118} control line for ten generations to isogenize the lines to the control line we would use in all of our mutant experiments. We also used a deficiency line (ED228Df) that has a ~700 kb deletion removing *SREBP*, *miR-33*, and just over 100 other genes.

Given the lipid phenotypes already reported for flies overexpressing *miR-33*, we first looked at TAG levels in adult flies lacking *miR-33*. Surprisingly, similar to the elevated TAG phenotype found when *miR-33* was overexpressed, we also found elevated levels of TAG normalized to protein in female adult flies carrying the large deletion ED228Df and either the $miR\text{-}33^{F11}$ or $miR\text{-}33^{KO}$ allele compared to ED228Df/+ controls when reared and maintained on our standard lab food

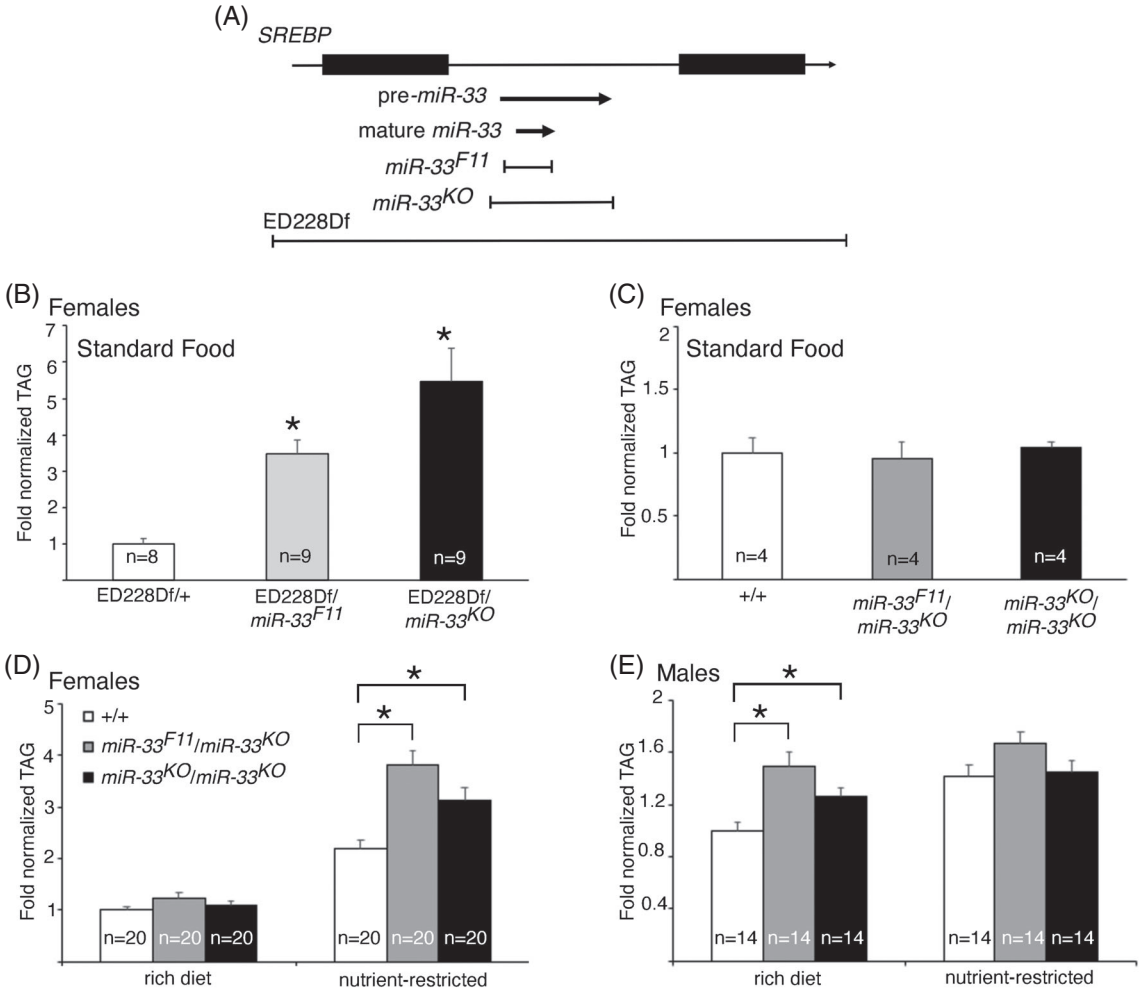

FIGURE 1 Flies lacking *miR-33* have elevated triacylglycerol (TAG) levels. A, Schematic showing location of *miR-33* locus within *SREBP* and the null alleles used in this study. Note that this shows a zoomed in view and does not show the entirety of the *SREBP* locus nor the extent of the ED228Df deletion, which removes about 100 genes in this region. B-E, Whole-body lysates from adult flies were measured for TAG and normalized to protein levels (mg TAG/mg protein). Mean normalized TAG is displayed, with error bars representing SEM. Two-tailed Student's *t* tests were conducted to compare each genotype/diet combination to control flies for that particular experiment. B, C, Adult mated female flies of the indicated genotypes were reared and then maintained as adults on our standard for 5 to 7 days before lysis. An asterisk indicates $P < .05$ compared to controls fed the same diet. D, E, Adult mated female (D) or male (E) flies were reared on nutrient-restricted food with or without yeast paste for 10 days. The data shown is the average of 4 to 5 independent experiments and the ages of the flies ranged from 12 to 19 days old

(Figure 1B). However, flies that were transheterozygous for the *miR-33*^{F11} and *miR-33*^{KO} alleles, as well as those homozygous for either allele, showed no consistent increase in normalized TAG levels compared to +/+ controls (Figure 1C). This suggests that there may be genetic interactions between the *miR-33* mutant alleles and genes removed by the large deletion, either the host gene, *SREBP*, or another locus included within the deletion.

To determine if diet might be influencing the phenotype in addition to other modifying genes, we reared female and male flies on our standard food and then, a few days after eclosion, we switched the adult flies for 10 days to either a nutrient-restricted diet lacking amino acids and lipids or a rich diet consisting of the nutrient-restricted diet enriched with yeast paste, before assaying for TAG levels (Figure 1D,E). The metabolic stress caused by a nutrient-restricted diet had previously been shown to cause an increase in fat accumulation in control female flies and also exacerbate the effects of loss of *miR-310s* on a range of phenotypes, including TAG accumulation.[24] Interestingly, the type of diet modified the effects of *miR-33* loss. On the nutrient-restricted diet, TAG levels were significantly higher in female *miR-33* mutants compared to controls, whereas these changes were not significant on the rich diet (Figure 1D). Curiously, the converse was true in male flies (Figure 1E), with a significant

difference only observed on the rich diet. Of note, the observed differences in normalized TAG levels in both females and males were most likely due to changes in TAG rather than changes in protein levels, as similar results were seen for TAG concentrations that were not normalized to protein (data not shown). Overall, in the absence of *miR-33*, the adaptive response to nutritional stress seems to be compromised in a context-dependent manner. This indicates that *miR-33* is regulating lipid homeostasis in adult flies.

2.2 | Loss of *miR-33* leads to increased resistance to starvation but increased sensitivity to oxidative stress

Given the impact of loss of *miR-33* on stored TAG in adults, we tested whether *miR-33*-deficiency affected survival under starvation conditions. We chose to perform these experiments on flies on our standard diet, where at baseline no consistent difference in TAG was observed. At ~10 days of age, we switched them to vials containing only a source of water (Figure 2A,B). Under these conditions, female mutants survived significantly longer with an increase in the average survival time of ~30% with a median life span of 145 hours for controls vs 187 hours for both mutant genotypes (P = 5.4e-7 in log rank test). Even though the shape of the curves for the two mutant lines was slightly different, survival was not significantly different between the two mutant genotypes (P = .21). Similar to females, male mutants also survived longer than controls with an almost 2-fold increase in average survival time (control life span = 86 hours, F11/KO = 150 hours, and KO/KO = 142 hours, P < 1.0e-10 in log rank test). In this case, there was also a small statistically significant difference between both mutant genotypes (P = .0488).

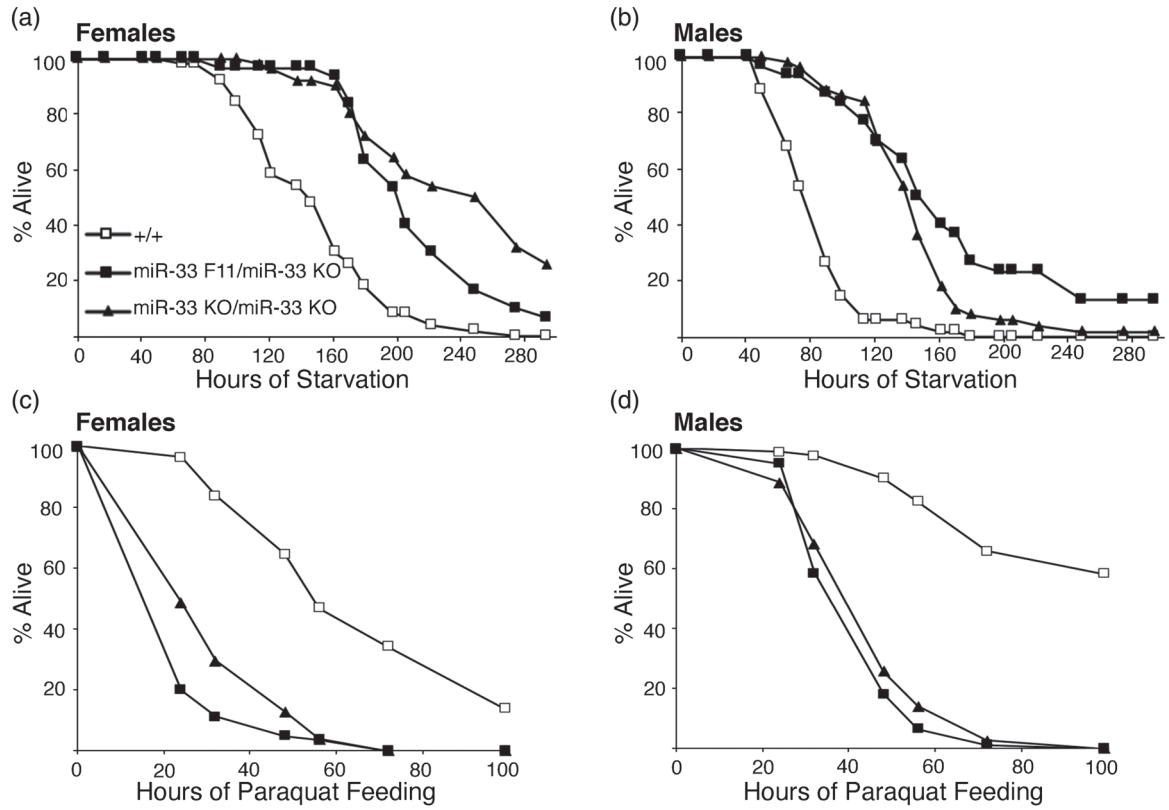

FIGURE 2 Loss of *miR-33* leads to increased resistance to starvation but increased sensitivity to oxidative stress. A, B, Adult 10- to 12-day-old females (A) and males (B) of the indicated genotypes were transferred to vials containing only a water soaked vial plug in the bottom of vial and monitored for survival. Thirty to fifty flies were monitored for each genotype/sex combination. C, D, Adult 4- to 7-day-old females (C) and males (D) of the indicated genotypes were starved overnight and transferred to vials containing only 5 mM paraquat in 1% (wt/vol) sucrose as a food source and monitored for survival. Seventy-seven to eighty flies were monitored for each genotype/sex combination. Results were analyzed using OASIS 2; mutants showed a statistically significant from controls in a log-rank test (P value <.00001) for both sexes exposed to each treatment

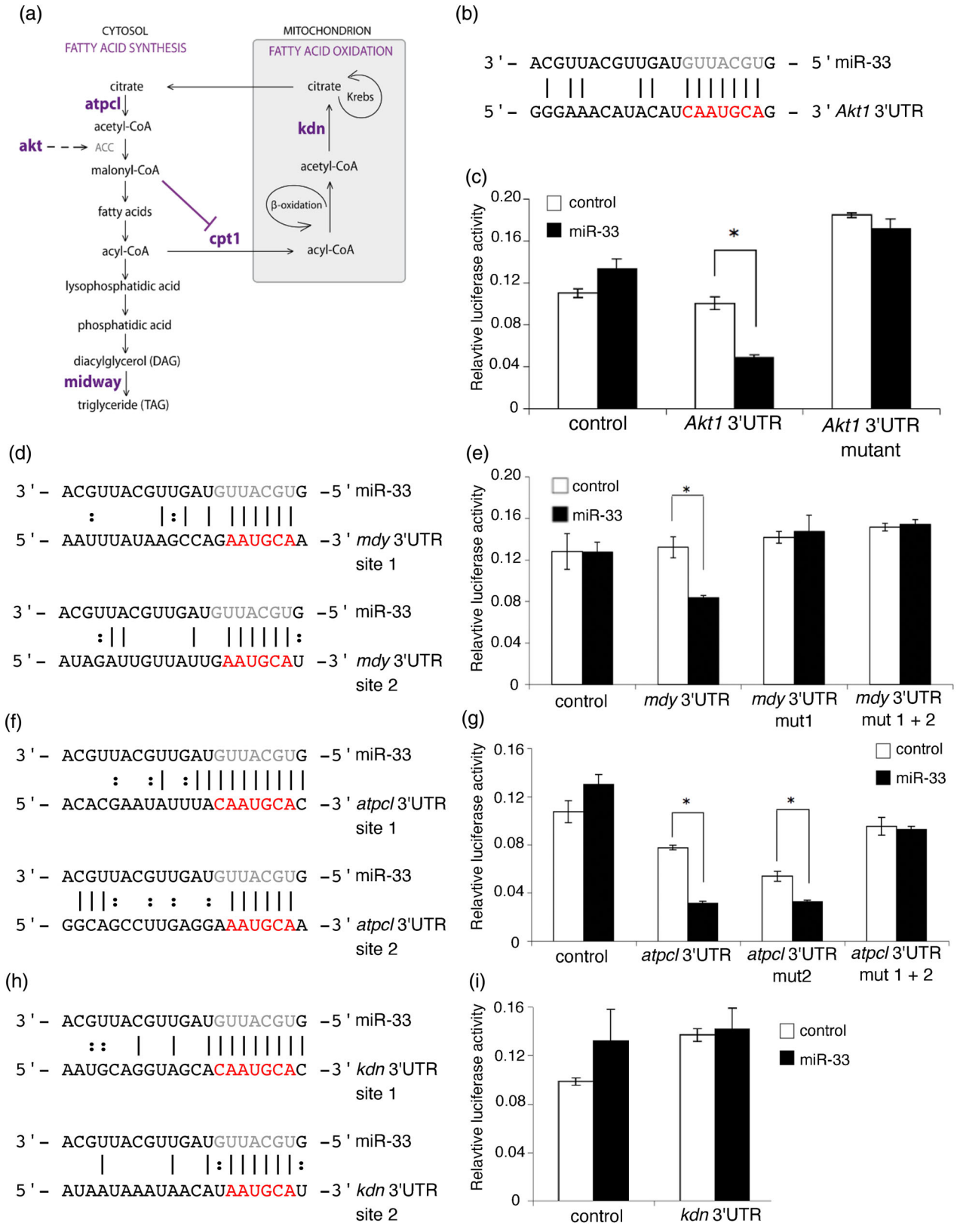

FIGURE 3 *miR-33* targets play a role in triacylglycerol (TAG) synthesis. A, Schematic representation of putative *miR-33* targets involved in TAG synthesis. B, D, F, H, Predicted binding sites of *Drosophila melanogaster miR-33* in the *Akt1* 3′UTR (B), *midway* (*mdy*) 3′UTR (D), *atpcl* 3′UTR (F), and the *kdn* 3′UTR (H). Sequences complementary to the *miR-33* seed sequence are shown in red. C, E, G, I, Reporter assays using the 3′UTR of the predicted target transcript, a mutant in which the predicted binding site or sites were inactivated (mutant, mut1, or mut2), or the parental pGL3-control plasmid (control) were performed in the presence of a synthetic fly pre-*miR-33* or a pre-miRNA control. Values are normalized to the activity of a constitutive *Renilla* luciferase expression construct and represented as mean ± SD of triplicates. An asterisk indicates $P < .05$ in a Student's *t* test (n = 3)

Flies fed a high fat diet, or that are genetically induced to have elevated insulin signaling, often show not only increased survival upon starvation but also increased sensitivity to oxidative stress.[25-29] To determine whether *miR-33* mutants show a similar combination of phenotypes, we exposed flies to paraquat, which leads to the production of reactive oxygen species in the mitochondrial respiratory chain.[30] In both males and females we saw a dramatic decrease in survival of *miR-33* mutants compared to controls (Figure 2C,D). These results are consistent with *miR-33* regulating metabolic processes in *D melanogaster*, possibly by targeting mRNAs encoding enzymes involved in these processes.

2.3 | Multiple *miR-33* targets play a role in TAG synthesis

We have previously reported that *miR-33* can target the 3′UTR of the *D melanogaster* ortholog of *Cpt1*, a positive regulator of fatty acid degradation via β-oxidation.[7] Loss of *miR-33* may consequently lead to an increase in Cpt1 levels. However, an increase in β-oxidation would be expected to decrease cellular lipid levels rather than increase them as observed in *miR-33* deficient flies. To understand how loss of *miR-33* might elicit the observed phenotypes, we went back to the list of potential targets of *miR-33* in *D melanogaster* and realized that it contained several additional genes directly or indirectly involved in lipid metabolism including *Akt1*, *midway* (*mdy*), *atpcl*, and *knockdown* (*kdn*) (Figure 3A). Notably, several of these predicted targets play a well-defined role in lipid synthesis, and thereby might contribute to the metabolic changes observed in *miR-33* deficient flies.

To determine whether *miR-33* could directly target these mRNAs, we generated reporter constructs for each target that contained the region of the 3′UTR with the putative *miR-33* binding sites behind a constitutively active firefly luciferase expression cassette (Figure 3B,D, F,H). In contrast to the control reporter construct that only contained the luciferase cassette, co-transfection of the construct containing the *Akt1* 3′UTR with synthetic pre-*miR-33* significantly reduced the luciferase activity compared to transfection with a control pre-miRNA (Figure 3C). Inactivation of the predicted seed binding site (highlighted in red in Figure 3B) by site-directed mutagenesis completely abolished this response, suggesting that *miR-33* can directly target the *Akt1* transcript through this site.

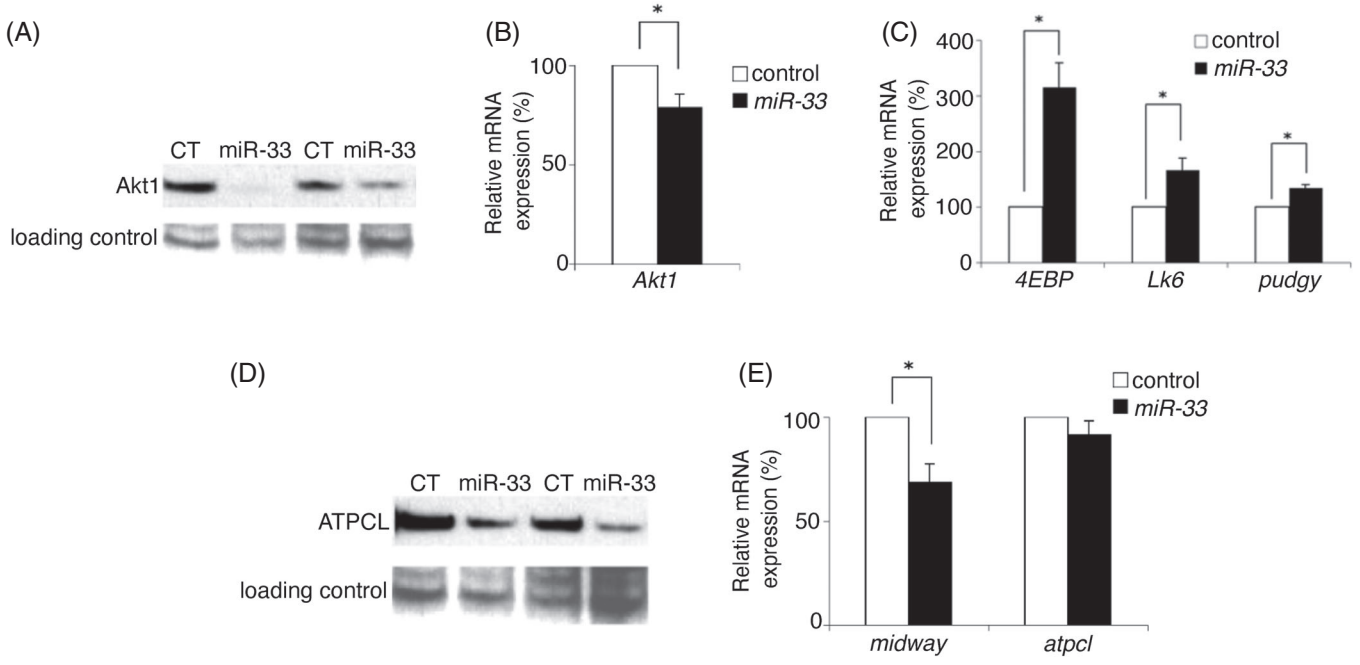

FIGURE 4 *miR-33* targets *Akt1*, *ATPCL*, and *midway* in vivo. A, D, Western blot analysis of Akt1 (A) and ATPCL (D) in fat bodies of 4- to 5-day-old virgin females expressing GFP alone (control: CT) or GFP and *miR-33* under the control of the yolk-*GAL4* driver. Samples are from two independent crosses. *Loading control* denotes a non-specific band recognized by an unrelated antibody, and is used to demonstrate even loading of samples. B, C, E, Quantitative RT-qPCR for *Akt1*, negative targets of insulin signaling (*4EBP*, *Lk6*, and *pudgy*), *midway*, and *ATPCL* relative to *gapdh* values in fat bodies of 4- to 5-day-old virgin females expressing GFP or GFP and *miR-33* under the control of the yolk-*GAL4* driver. Values represent means and SE obtained in six independent experiments, where the expression levels were normalized to values obtained in controls expressing GFP alone. An asterisk denotes $P < .05$ in a Student's *t* test

The 3′UTRs of *mdy*, *atpcl*, and *kdn* contain two putative *miR-33* binding sites (Figure 3D,F,H). Transfection of a synthetic pre-*miR-33* caused a decrease in luciferase activity for the *mdy* and *atpcl* reporter constructs but not *kdn*, suggesting that *mdy* and *atpcl* are targets of *miR-33* but that *kdn* likely is not (Figure 3E,G,I). Although mutation of the predicted seed binding sites abolished the response in the *mdy* and *atpcl* reporters, not all predicted binding sites seemed to contribute to the response of *miR-33* to the same extent (Figure 3D,F,H). For example, deletion of site 1 in the *mdy* transcript completely abolished the reporter response to *miR-33*. These results suggest that *miR-33* can directly target multiple mRNAs involved in triglyceride synthesis.

2.4 | *miR-33* targets *Akt1*, *atpcl*, and *midway* in vivo

To confirm that *miR-33* can target *Akt1*, *atpcl*, and *midway* in vivo, we performed western blots and RT-qPCR on dissected fat bodies from adult female flies overexpressing *miR-33* to examine protein and mRNA levels of these targets, respectively (Figure 4). Akt1 protein and mRNA levels were both inhibited by *miR-33* (Figure 4A, B). To determine whether the decrease in Akt1 protein might have an impact on downstream signaling, we assayed the expression of three targets of FOXO that are predicted to increase with an decrease in Akt1 activity, and subsequent inhibition, of FOXO.[31-33] Overexpression of *miR-33* caused the predicted increase in *4EBP*, *Lk6*, and *pudgy* suggesting that this change in Akt1 levels can lead to a change in downstream signaling (Figure 4C). We also found a decrease in ATPCL protein but no significant change in its mRNA when *miR-33* levels were elevated (Figure 4D,E). As no antibody was available to assay Midway protein, we were able to only look at *midway* mRNA and found that *miR-33* also reduced *midway* mRNA levels in vivo (Figure 4E). Together with the reporter assays, these results suggest that *miR-33* can target these mRNAs in vivo, most likely through binding sites in their 3′UTRs, and this targeting may contribute to changes in triglyceride levels and sensitivity to starvation and oxidative stress.

2.5 | Loss of *miR-33* results in increased abdominal cuticle pigmentation

We have previously shown that nutritional state can regulate adult cuticle pigmentation and that insulin signaling through Akt1 positively regulates pigmentation in *D melanogaster*.[34] Upon careful observation of the *miR-33* mutants, we noticed that the most posterior abdominal cuticle of the female mutants appeared to have more pigmentation (Figure 5A-D). When quantified, we found that a larger area of the abdominal A5 and A6 segments are pigmented in *miR-33* mutants compared to controls, with a stronger effect in the A6 segment (Figure 5E). These results were consistent whether the mutants were homozygous for either allele or transheterozygous, with one copy of each mutant allele. This suggests that *miR-33* serves as a negative regulator of pigmentation by limiting the segment area containing pigment in female *D melanogaster*.

To determine whether this increase in pigmentation is due to loss of *miR-33* specifically in the developing cuticle, we used the GAL4/UAS transgenic system to inhibit *miR-33* with a "sponge." This transgene expresses, under the control of the UAS enhancer, an mRNA encoding mCherry with a 3′UTR that contains multiple copies of a *miR-33* binding site.[35] We used the pnr-*GAL4* transgene to drive expression of the GAL4 transcription factor along the dorsal midline of the developing fly, in the epidermal cells underlying the secreted cuticle[36]; GAL4 then turns on expression of the *miR-33* sponge to inhibit *miR-33* in those cells (Figure 5F,G). To increase activity of the GAL4 transcription factor and transgene expression, these flies were reared at 28°C. In these experiments, it needs to be noted that this elevated temperature leads to decreased pigmentation in flies reared at this higher temperature.[37] When comparing our experimental groups, we saw an increase in the area of pigmented cuticle, most notably in the A6 segment, in flies expressing the *miR-33* sponge compared to those expressing a control sponge with scrambled microRNA binding sites (Figure 5G vs F). This suggests that *miR-33* is regulating cuticle pigmentation directly in the cuticle and not through indirect effects.

2.6 | Decrease in pigmentation caused by overexpression of *miR-33* in the developing cuticle

To determine whether *miR-33* is indeed a negative regulator of pigmentation, we overexpressed *miR-33* using the pnr-*GAL4* driver (Figure 6). We first used a UAS-*miR-33* line that expresses both DsRed and *miR-33*. Overexpression of *miR-33* with the pnr-*GAL4* driver led to a decrease in pigmentation in both the A5 and A6 segments, most visible in the A6 segments (Figure 6D vs A-C and E). This loss was only present in flies containing both the UAS-*miR-33* and pnr-*GAL4* transgenes. We observed very similar changes when we used a second, separately derived UAS-*miR-33* line that only expresses

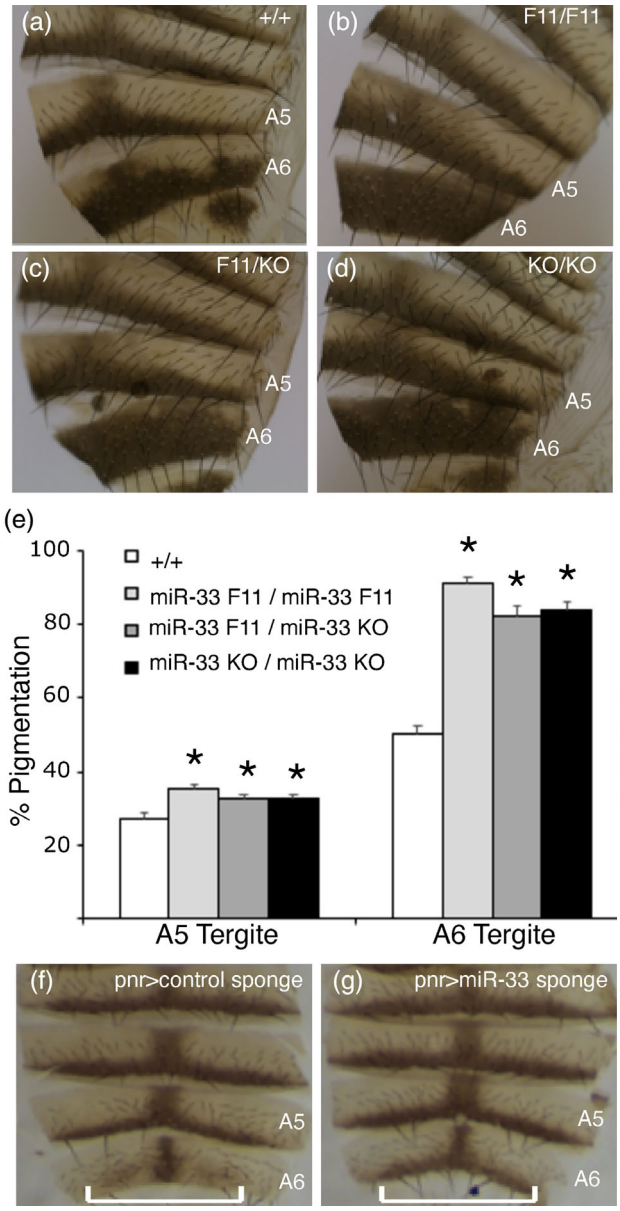

FIGURE 5 Loss of *miR-33* results in increased abdominal cuticle pigmentation. A-D, F, G, Dorsal abdominal cuticles from 3- to 5-day-old *Drosophila melanogaster* adult females. All flies contain the w^{1118} mutation and were reared at 25°C (A-D) or 29°C (F, G). The dorsal midline is towards the left of each panel (A-D) or in the center (F, G). The A6 tergite of wild-type (+/+) flies (A) and from flies that are homozygous for the $miR\text{-}33^{F11}$ null mutation (B), transheterozygous for the $miR\text{-}33^{F11}$ and $miR\text{-}33^{KO}$ mutations (C) or homozygous for the $miR\text{-}33^{KO}$ mutation (D). To control for genetic background, both $miR\text{-}33^{F11}$ and $miR\text{-}33^{KO}$ lines were backcrossed to the w^{1118} flies for 10 generations. E, Percent pigmentation of the A5 and A6 segments were determined by analyzing 10 cuticles for each of the indicated genotypes. Mean percent pigmentation is displayed, with error bars representing SEM. Two-tailed Student's *t* tests were conducted to compare each mutant to wild-type and an asterisk indicates $P < .05$. F, G, The pnr-*GAL4* transgene was used to drive expression along the dorsal midline during development of a transgene expressing mCherry RFP with 20 copies a scrambled microRNA binding site (F, control sponge) or the *miR-33* binding site (G, miR-33 sponge) in its 3′UTR. The white bracket indicates the approximate pnr-*GAL4* expression region

miR-33 (without DsRed) (Figure 6F,G). In addition, using a GAL4 driver that expresses in a similar pattern to the *yellow* gene, with greatest expression towards the posterior end of each segment (y-*GAL4*), we confirmed the loss of pigmentation with overexpression of *miR-33* (Figure 6H,I). These results confirm that *miR-33* is acting directly in the developing abdominal cuticle in female flies to inhibit pigmentation.

Given that *Akt1*, a known positive regulator of pigmentation, can be targeted by *miR-33* we set out to determine whether the loss of pigmentation caused by overexpression of *miR-33* might be due, at least in part, to the loss of *Akt1*. We used a UAS-*Akt1* transgene that lacks most of the endogenous 3′UTR of *Akt1*, including the *miR-33* binding site. Expression of Akt1 resulted in an increase in pigmentation compared to control, consistent with our previous findings that Akt1 increases pigmentation (Figure 6J vs B). However, co-expression of Akt1 with *miR-33* did not prevent the decrease in pigmentation seen with overexpression of *miR-33* alone (Figure 6K vs D). These findings suggest that *miR-33* is likely not acting through *Akt1* to inhibit cuticle pigmentation but is instead acting through one or more other targets, potentially downstream of Akt1.

2.7 | Loss of *miR-33* leads to decreased interfollicular stalk cells in the adult ovary

Fat body lipid storage and nutritional status are important for *Drosophila* germ cell function, oogenesis and, consequently, fertility. Each female *Drosophila* contains two ovaries made up of ~18 ovarioles, which contain egg chambers developing from the germarium of progressively later stages up to Stage 14, the mature egg (Figure 7A).[38] The germarium contains germline stem cells and somatic/follicle stem cells, which contribute to various specialized cell types within the ovariole and egg chamber. Typically, an egg chamber contains 16 germ cells: 15 nurse cells and one oocyte. Egg chamber formation involves three interdependent processes, (a) the proliferation of somatic stem cells (follicle cells), (b) follicle cell migration and envelopment of the 16-cell germline cyst, and (c) follicle cell differentiation to generate stalk cells.[39] Issues with these processes are associated with egg chamber abnormalities including two or more complete germline cysts in one egg chamber, split cysts (one egg chamber with too few germ cells and another with too many), and a single epithelial bag of cysts in an ovariole.[39] A large-scale screen of microRNAs found no change in fertility in $miR\text{-}33^{KO}/ED228Df$ females, but they did report a partially penetrant loss of interfollicular stalk cells, abnormal germ cell number, and abnormal encapsulation during oogenesis in *miR-33* mutants, with

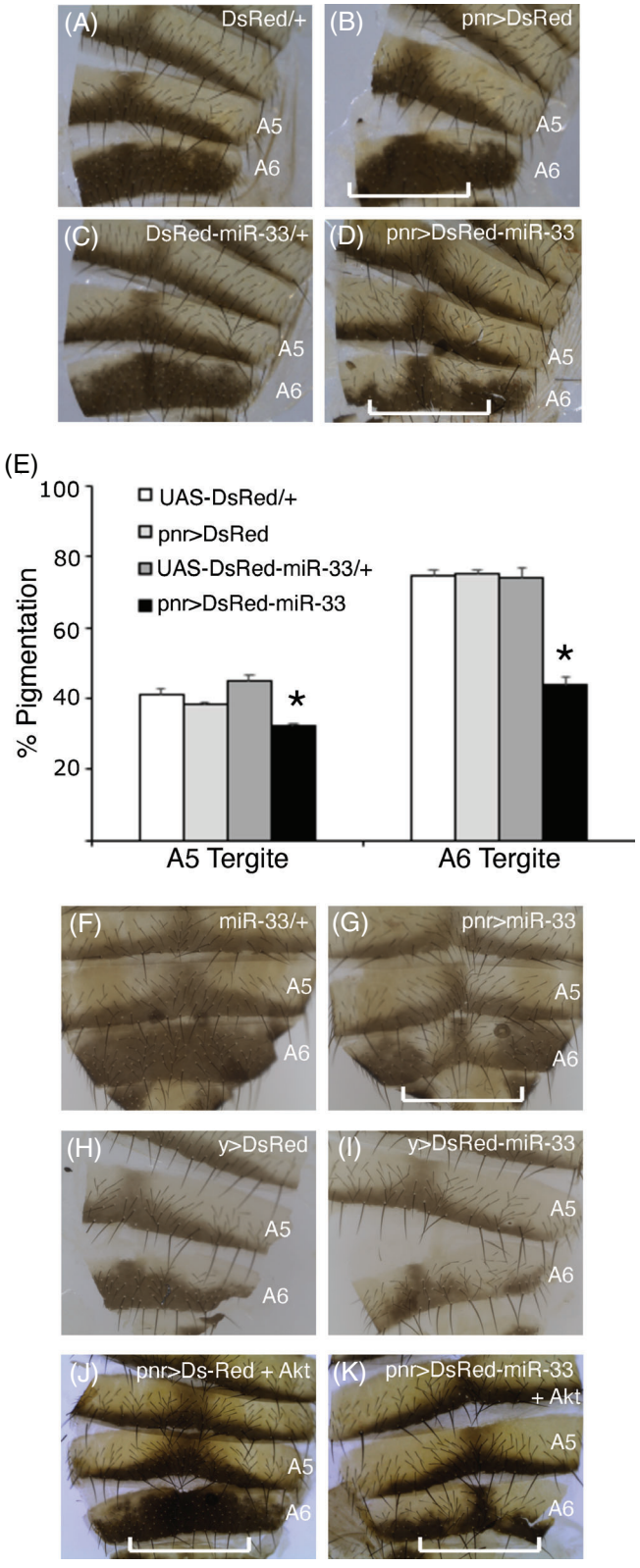

FIGURE 6 Decrease in pigmentation caused by overexpression of *miR-33* in the developing cuticle. A-D, F-I, Dorsal abdominal cuticles from 3- to 5-day-old *Drosophila melanogaster* adult females. All flies contain the w^{1118} mutation and were reared at 25°C. The dorsal midline is towards the left of each panel. The pnr-*GAL4* transgene was used to drive expression along the dorsal midline during development. The white bracket indicates the approximate pnr-*GAL4* expression region. Flies containing the UAS-*DsRed* transgene alone (A) or in combination with the pnr-*GAL4* driver (B) or the UAS-*DsRed-miR-33* transgene alone (C) serve as controls for those that contain both the UAS-*DsRed-miR-33* and pnr-*GAL4* transgenes (D). E, Percent pigmentation of the A5 and A6 segments were determined by analyzing 10 cuticles for each of the indicated genotypes. Mean percent pigmentation is displayed, with error bars representing SEM. Two-tailed Student's *t* tests were conducted to compare each mutant to compare each genotype to flies containing only the UAS-*DsRed* transgene for A5 and A6 tergites. An asterisk indicates $P < .05$. F, G, Flies containing the independently generated UAS-*miR-33* transgene alone (F) or in combination with the pnr-*GAL4* transgene (G). H, I, The y-*GAL4* transgene was used to drive expression in the pattern of the *yellow* gene, in the posterior region of each abdominal tergite. Flies contained the y-*GAL4* driver along with the UAS-*DsRed* transgene (H) or the UAS-*DsRed-miR-33* transgene (I). J, K, Flies containing a UAS-*Akt1* transgene and pnr-*GAL4* with the *DsRed* control transgene (J) or the UAS-*DsRed-miR-33* transgene (K)

penetrance of the defects likely dependent on dietary conditions and age.[21] We decided to follow up on this earlier finding, and quantify the ovary defects in our *miR-33* mutant age-matched females kept in constant, non-crowded conditions.

We reared and maintained control and *miR-33* females on our standard lab diet in carefully controlled conditions to limit overcrowding and allow flies ample access to food. We also carefully controlled the age of the flies that were analyzed. Consistent with the previous report, we found a loss of stalk cells with loss of *miR-33* alone (Figure 7B). Specifically we found that this loss is observed by Stage 2 with no further loss in the remaining stages of oogenesis; beginning at Stage 2 *miR-33* mutants have on average two fewer stalk cells at all stages compared to controls. This suggests that *miR-33* is required for the specification or survival of stalk cells at early stages of oogenesis. However, we did not find a consistent, significant difference in the number of germ cells in *miR-33* mutant chambers (Figure 7E) nor obvious defects in encapsulation (data not shown).

To determine whether the decrease in stalk cells in *miR-33* mutants was due to loss of *miR-33* in stalk cells, we expressed the *miR-33* sponge construct using the 109-53-*GAL4* driver, which drives expression in the stalk cells, terminal filament, cap cells and muscle sheath cells of the ovary.[40] The number of stalk cells at each of the stages tested was unaffected by expression of the *miR-33* sponge, suggesting a non-cell autonomous role for *miR-*

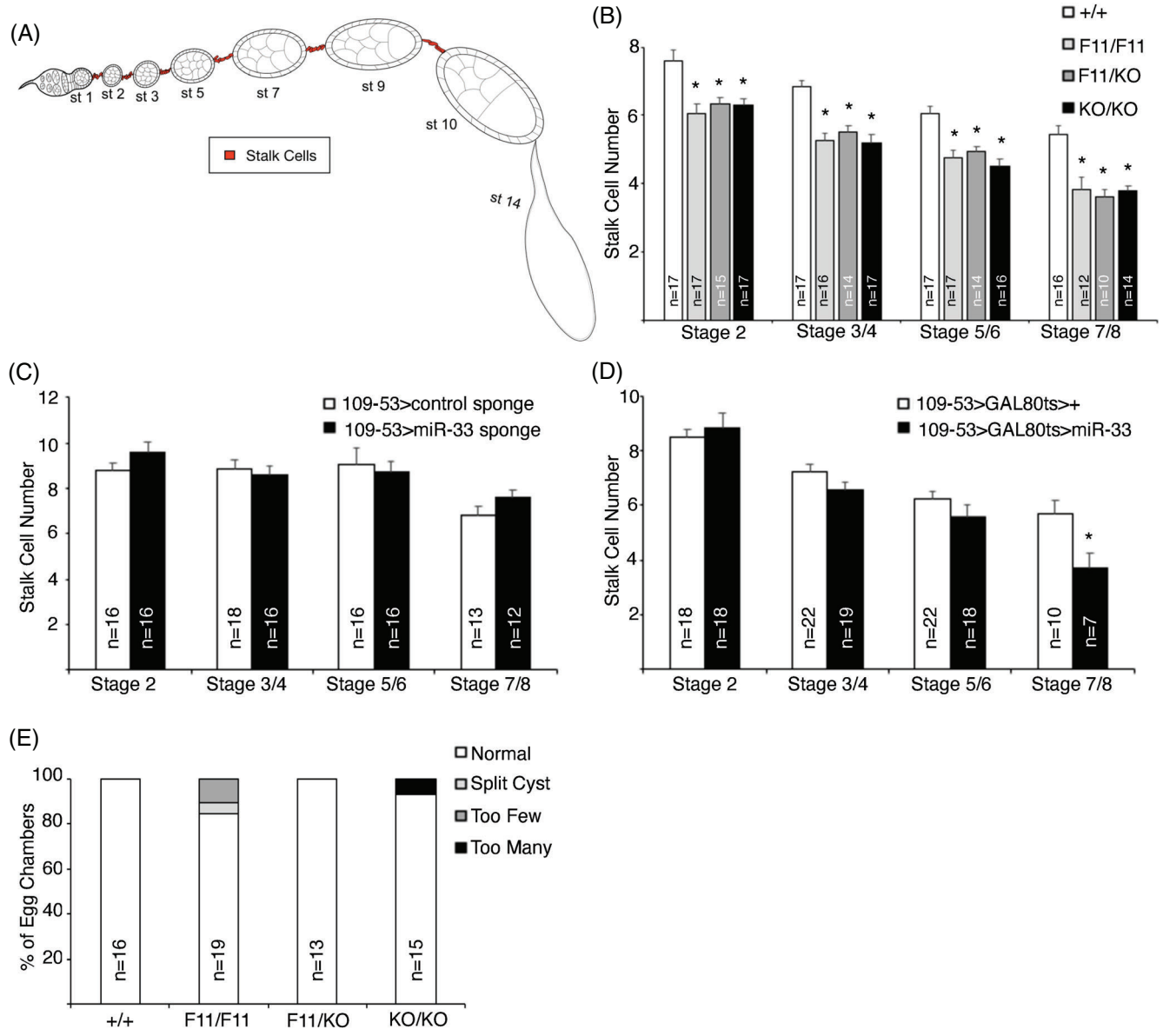

FIGURE 7 Loss of *miR-33* leads to decreased interfollicular stalk cells without impacting germ cell number. A, Schematic of oocyte development and location of interfollicular stalk cells. B, Stalk cell number was determined in 3- to 4-day-old adult females of the indicated genotype using confocal imaging of egg chambers stained for a marker of stalk cells, Lamin C. Mean number of cells is displayed with error bars representing SEM. The sample size for each stage/genotype combination is indicated. An analysis of variance (ANOVA) was performed to compare mutant genotypes to wild-type at each stage and an asterisk indicates $P < .05$. C, Stalk cell number was determined in 3- to 4-day-old females expressing the miR-33 or control sponge transgene specifically in the stalk cells using the 109-53-*GAL4* driver. Confocal imaging of egg chambers and analysis was similar to that described in B. ANOVA analysis found no statistically significant difference between control sponge and *miR-33* sponge for any Stage. D, Stalk cell number was determined in 6- to 11-day-old adult females overexpressing *miR-33* specifically in the stalk cells only during adulthood using a combination of the 109-53-*GAL4* and tub-*GAL80ts* transgenes, confocal imaging of egg chambers and analysis as in B. ANOVA analysis only found a statistically significant difference in stalk cell numbers between controls and *miR-33* overexpressors at Stage 7 to 8. E, Germ cell number was determined by confocal microscopy in the indicated number of ovarioles from 3- to 4-day-old females of the indicated genotypes

33 in regulating stalk cell number (Figure 7C). To further test the role of *miR-33* in stalk cells, we overexpressed *miR-33* using the 109-53-*GAL4* driver. Unfortunately, we recovered no adult female flies overexpressing *miR-33* using this driver, potentially due to lethality caused by expression in other tissues during development. To control the timing of GAL4 driven gene expression in these flies we added a transgene ubiquitously expressing a temperature sensitive negative regulator of GAL4, GAL80^{ts} (tub-*GAL80^{ts}*), such that GAL80 would be active

throughout development to adulthood and then switched the temperature after eclosion to release GAL80 inhibition of GAL4 and trigger expression of *miR-33*. Flies overexpressing *miR-33* in stalk cells as adults had similar numbers of stalk cells as control flies during earlier stages but had a loss of stalk cells by Stage 7/8 (Figure 7D). Together these findings suggest a possible non-cell autonomous effect of *miR-33* on stalk cell specification and/or maintenance, at least at early stages of oogenesis.

3 | DISCUSSION

Given previous findings that *D melanogaster miR-33* can target the ortholog of *Cpt1* in flies and that overexpression of *miR-33* can increase TAG levels in both larval fat body and adult,[7,12,20] we were initially surprised that loss of *miR-33* causes flies to be pre-disposed to elevated TAG levels. As a possible explanation, we found evidence that *miR-33* can also target the 3′UTRs of *atpcl*, *midway*, *and Akt1*, suggesting that an imbalance between TAG production and degradation is most likely causing the observed elevated TAG levels. Atpcl and Midway are directly involved in TAG synthesis. Atpcl (coding for ATP citrate lyase) uses citrate and ATP to generate cytoplasmic acetyl-CoA that is required for fatty acid synthesis, and Midway is the diacylglycerol acyltransferase mediating the last step of TAG synthesis.[41,42] Knockdown of *midway* by RNAi in cell lines prevents TAG accumulation.[43] In the case of Akt1, the mechanism is more indirect and likely involves effects on both fatty acid synthesis and degradation. In fact, Akt unleashes the activity of acetyl CoA carboxylase (ACC) by preventing the inhibitory phosphorylation by AMPK,[44] thereby increasing fatty acid synthesis. In addition, the product of ACC, malonyl CoA, has the potential to inhibit β-oxidation.[45,46] In vivo, adult fat body specific overexpression of an activated form of the insulin receptor, which acts upstream of Akt, has been reported to increase TAG levels.[47] The predicted increase in Atpcl, Midway, and Akt proteins resulting from loss of *miR-33* might thus contribute to increased fatty acid synthesis and reduced β-oxidation, resulting in more accumulation of TAG. However, we cannot rule out that there are other targets responsible for this and the other phenotypes in *miR-33* mutants.

Of note, the loss of *miR-33* alone did not lead to a consistent increase in TAG levels when they were reared and maintained on our standard laboratory food. Yet, we saw a consistent increase when flies contained one copy of either *miR-33* mutant allele and one copy of the larger ED228Df deficiency that removes ~100 genes, including *SREBP* and *miR-33*. This suggests that the large deletion allele includes genetic modifiers, either *SREBP* itself or one of the other genes, that contribute to enhancement of the phenotype. We also saw slight differences between the impacts of the two alleles (F11 vs KO) in the triglyceride assays and resistance to starvation and oxidative stress. Even though we backcrossed these lines 10 times to the line used as the control for these experiments, remaining differences in genetic background might explain these slight differences between lines, consistent with the hypothesis that genetic modifiers can influence the phenotype caused by loss of *miR-33*.

Although we saw no difference in TAG levels in flies only lacking *miR-33* on our standard diet, we observed sex-dependent differences upon metabolic stress. In female flies, we found slightly increased TAG in *miR-33* mutants that were fed a carbohydrate-only diet, but no difference on a rich diet, where yeast paste was added. Curiously, in male flies, we found the opposite phenotype to females, with an increase in TAG with the rich diet but no change on the nutrient restricted food. The difference between the response of females and males to the change in diet is consistent with other studies showing that there is metabolic sexual dimorphism in *D melanogaster*.[48] Studies have found the metabolomes to be surprisingly different between adult females and males, which may be consistent with the differing reproductive needs of the two sexes.[49-51] Of most relevance to this current study, TAG metabolism is strikingly different between the sexes, with differences in TAG levels between adult females and males emerging within 5 days post-eclosion, and a greater decrease in TAG in males than in females upon starvation, suggesting differences in both synthesis and breakdown in TAG between sexes.[52] Interestingly, *midway* and other genes involved in regulating TAG homeostasis are sex biased, with *midway* being expressed more in males, but with a more pronounced expression increase in females in response to starvation. One possible contributor to these sex differences might be the elevated levels of the hormone Ecdysone found in females compared to males.[48] Consistent with this idea, the differences in TAG levels between females and males were diminished by loss of the ecdysone receptor (EcR) in the CNS, suggesting a role for this hormone in systemically regulating energy metabolism.[53] Given that EcR promotes expression of *SREBP*,[19] the host gene for *miR-33*, the possible role of *miR-33* in contributing to these sex-dependent metabolic differences is an interesting future direction of research.

Interestingly, this predisposition of *miR-33* mutant flies to increased levels of triglyceride storage in the face of metabolic stress is reminiscent of the phenotype of *miR-33* mutant mice[8,15,16] and suggests a conserved role for *miR-33* in preventing extremes in cellular lipids in

response to environmental fluctuations, albeit through non-conserved targets. Consistent with this hypothesis, the predisposition to increased TAG, along with increased survival under starvation conditions and increased sensitivity to oxidative stress we see in *miR-33* mutants is similar to wild-type flies on a high fat diet.[25-28,54] This combination of phenotypes is also consistent with an increase in insulin signaling, similar to that found in larvae with elevated insulin signaling targets due to loss of the downstream effectors FOXO or 4EBP.[29] Surprisingly, we found resistance to starvation even when flies were fed our standard diet, a situation in which we did not see a difference in stored TAG levels. Although we do not have an explanation for this, we note that our TAG measurements may have been limited in sensitivity, especially given that we assayed TAG from only whole animals and did not conduct further analysis of the differences between storage sites across the body which might be more relevant to responses to starvation. We also cannot rule out the possibility that there are other yet to be identified effects of loss of *miR-33* on metabolic state, beyond impacts on TAG levels, that are contributing to resistance to starvation in mutants.

We also found evidence that *miR-33* is an inhibitor of abdominal pigmentation in female adult *D melanogaster*. Typically, pigmentation in *D melanogaster* females shows much more variation than in males and is responsive to a variety of environmental signals, including temperature and diet.[37,55] Yet, rather than impacting pigmentation indirectly through a systemic effect on metabolism, two lines of evidence indicate that *miR-33* is likely acting directly in the epidermal cells underlying the secreted cuticle to inhibit pigmentation. First, expression of the *miR-33* sponge in the developing cuticle leads to an increase in pigmentation. Second, overexpression of *miR-33* in the developing cuticle leads to a decrease in pigmentation. This indicates that pigmentation changes observed in *miR-33* mutants might also be caused by the loss of *miR-33* in the epidermal cells that secrete the adult cuticle. Of relevance to this study, we and others have previously identified nutritional status to be a potent regulator of pigmentation in *D melanogaster* and that insulin signaling through Akt1 can positively regulate pigment production alongside the Tor pathway, possibly through regulation of Tyrosine Hydroxylase, the rate limiting enzyme in melanin biosynthesis.[34,56] However, we observed that Akt1 cannot rescue the loss of pigmentation caused by overexpression of *miR-33*. This indicates that *miR-33* is acting through another target to regulate pigmentation. The other targets we have reported in this study have no known role in pigmentation, so the relevant target is likely one that has not yet been identified.

A large-scale study of microRNA function in *D melanogaster* reported that flies lacking *miR-33* displayed an incompletely penetrant change in interfollicular stalk cell numbers as well as germ cell numbers and defects in encapsulation of germ cells by follicle cells.[21] By controlling female age and growth conditions, we found a subtle decrease of, on average, two stalk cells beginning at the first stage we examined of oogenesis, Stage 2, with no signs of further decrease beyond the loss of two stalk cells as oogenesis progressed. Yet, we did not find any change in germ cell number or signs of encapsulation defects. One possible explanation for the difference in our findings is that, in the previous study, they only examined flies that carried the large ED228Df deficiency over the *miR-33* mutant allele. Perhaps, similar to TAG levels, heterozygosity at another locus within the deletion also enhances this phenotype in *miR-33* mutants. With regard to the reduction in stalk cells, we hypothesize that the effect is likely due to the loss of *miR-33* in cells other than the stalk cells themselves based on our finding that neither inhibition of *miR-33* in stalk cells with the *miR-33* sponge, nor overexpression of *miR-33* specifically in stalk cells, had an effect on stalk cell numbers at those earlier stages. We must note, however, that we cannot rule out the possibility that the *miR-33* sponge had no effect on stalk cell numbers because it was not able to completely inhibit *miR-33* as fully as occurs when *miR-33* is removed through mutation.

The decreased number of stalk cells at the later stage of oogenesis caused by *miR-33* overexpression is surprising because we also see a decrease in *miR-33* mutants, but at earlier stages. Thus, the mechanism underlying these similar yet distinct phenotypes with gain and loss of *miR-33* may be different. The known regulators of stalk cell numbers, such as JAK/STAT signaling, regulate stalk cell numbers beginning at earlier stages rather than late.[40] Interestingly, one of the *miR-33* targets we have identified in this study, *midway*, is known to regulate oogenesis, and its loss causes premature apoptosis of nurse cells during later stages of oogenesis and results in infertility.[42] The role Midway plays, if at all, in stalk cell maintenance and survival at either early or late stages has not yet been reported. Likewise, whether the expected changes in Midway due to changes in *miR-33* levels might affect stalk cell numbers is currently unknown and warrants further study.

With regard to the possibility that *miR-33* is acting non-cell autonomously to positively regulate stalk cell numbers, previous studies have found that signals from another somatic cell type in the ovary, polar cells, influence stalk cell number, including through secretion of Upd, a ligand that activates JAK/STAT signaling in stalk cells to inhibit apoptosis.[40,57,58] Loss of stalk cells could

result if polar cell specification is altered or their release of signaling molecules to stalk cells are reduced by loss of *miR-33*. Future studies warrant investigating the role of *miR-33* in polar cells and whether follicle cell specification and proliferation is altered in the adult germarium.

Given our findings that *miR-33* leads to signs of systemic metabolic dysfunction, including altered lipid homeostasis, one possible explanation for the defects seen in stalk cells could be changes in signaling pathways that are responsive to metabolic state. A previous study found that the *miR-310s* act through the targeting of multiple Hedgehog pathway components to adjust and adapt signaling in the ovary when dietary conditions change.[24] Although the mechanisms underlying the pleiotropic action of *miR-33* still need to be worked out, our study presents intriguing preliminary findings that suggest *miR-33* may play a role in fine-tuning adaptive metabolic responses that in turn impact multiple phenotypes in a variety of tissues.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fly stocks and food

All stocks contained the w^{1118} null mutation in the *white* gene, and flies were reared at 25°C unless otherwise noted. The following lines were acquired from the Bloomington *Drosophila* Stock Center at Indiana University: ED228DF (#8086), pnr-*GAL4* (#3039), y-*GAL4* (#44267),UAS-*miR-33* (#59871), UAS-control sponge (#61501), UAS-*miR-33*-sponge (#61385), UAS-Akt1 (#8191), tub-$GAL80^{ts}$ (#7019) and 109-53-*GAL4* (#7025). UAS-*DsRed* (P49) and UAS-*DsRed-miR-33* (7170) stocks were kindly provided by Dr. Eric Lai and Dr. Justin DiAngelo provided the Yolk-*GAL4* stock. The $miR\text{-}33^{F11}$ is a null allele that was generated using CRISPR/Cas9 editing. Embryos expressing Cas9 in the germline (vas-*Cas9*; Bloomington stock #55821) were injected by Bestgene Inc. with two pU6-BbsI-chiRNA constructs engineered to express gRNAs that would cause double-stranded breaks on either side of the region that gives rise to the predicted mature *miR-33*. Progeny of injected embryos were screened for deletions, and the $miR\text{-}33^{F11}$ line has a 53 bp deletion that removes the mature *miR-33* sequence along with some of the pre-miRNA sequence. The $miR\text{-}33^{KO}$ null mutation was previously reported (Bloomington #58930) and contains a 134 bp deletion that removes all of the pre-miR-33 and some extra intronic sequence.[21] Properly spliced *SREBP* mRNA transcript expression was confirmed in both mutant lines. Both mutant lines were backcrossed to w^{1118} control flies for over ten generations to isogenize the stocks. The pU6-BbsI-chiRNA parental plasmid was a gift from Melissa Harrison, Kate O'Connor-Giles, & Jill Wildonger (Addgene plasmid 45 946).[23]

Unless otherwise noted, flies were reared and maintained on our standard lab food (10% glucose, 10% Brewer's yeast, 1.4% Agar plus Tegosept and propionic acid). For the nutrient restricted condition in Figure 1, flies were transferred from standard food to nutrient-restricted food (2% Agar, 25% apple juice, and 2.5% sucrose) and reared on this diet for 10 consecutive days at 25°C prior to TAG and protein assays. The non-nutrient-restricted (rich) condition was performed in parallel with nutrient-restricted food that was supplemented with yeast paste (1.5 g dry yeast in 2.5 mL 5% propionic acid). In all instances, flies were transferred to fresh food every 2 days and kept under similar, non-crowded conditions.

4.2 | TAG and protein quantification

Two adult mated females or three adult mated males were collected on ice in 250 μL of buffer containing 140 mM NaCl, 50 mM Tris/HCl (pH 7.4), 0.1% Triton X-100 and a protease inhibitor cocktail (Roche complete). The flies were briefly sonicated and clarified by centrifugation at maximum speed. Protein concentrations were measured using the BCA Protein Assay Kit (Pierce) and TAG concentrations were measured using the Triglyceride Liquicolor assay (Stanbio) according to the manufacturer's instructions.

4.3 | Starvation and paraquat sensitivity assays

For the starvation assays, adult female and male flies were reared on standard food for 10 to 12 days after eclosion, then transferred to vials containing only a water-soaked Flug vial closure (Genesee Scientific) in the bottom of each vial at a density of 10 flies per vial and monitored for survival. For the paraquat sensitivity assays, adult female and males flies were reared on standard food for 4 to 7 days, then transferred to vials containing water-soaked filter paper overnight at a density of 10 flies per vial. The next day, the 5- to 8-day-old flies were transferred to vials containing filter paper soaked with 5 mM paraquat in a 1% (wt/vol) sucrose solution and monitored for survival. Results were analyzed using the Online Application for Survival Analysis 2 (OASIS 2).[59]

4.4 | 3′UTR reporter assays

Genomic regions encompassing the predicted *miR-33* binding sites in the 3′UTR of *D melanogaster Akt1*,

midway, *atpcl* and *kdn* were amplified and inserted downstream of the constitutively active luciferase expression cassette of the plasmid pGL3 control (Promega). Seed binding regions of predicted binding sites (indicated in red) were replaced by restriction sites to introduce site-directed mutations (for *Akt1* by BglII; for *midway* by BglII and HindIII; for *atpcl* by BglII and HindIII). Synthetic *D melanogaster* pre-*miR-33* and a negative pre-miRNA control were obtained from Life Technologies. Transfections of 293T cells were performed in 24-well plates in 0.5 mL with Lipofectamine 2000 (Thermo Fisher Scientific) using pre-miRNAs at a final concentration of 3.3 nM as well as 200 ng of firefly luciferase constructs and 50 ng of a constitutively active *Renilla* luciferase construct (Promega). Relative luciferase activity in all figures refers to firefly luciferase activity normalized to *Renilla* luciferase activity.

4.5 | Western blot

Fat bodies from 4- to 5-day-old virgin female flies were dissected in PBS and eight or more dissected abdomens were lysed in 250 μL RIPA buffer containing protease and phosphatase inhibitors (Roche). Lysates were briefly sonicated and cleared by centrifugation. Protein concentrations were determined by the BCA protein Assay Kit (Pierce) and equal amounts of protein were resolved on 10% Bis-Tris polyacrylamide mini-gels and transferred to PVDF membranes (Immobilon P, Millipore). Equal transfer was validated by staining membranes with Ponceau Red (Sigma). Primary antibodies were used at the following dilutions: rabbit anti-ATPCL at 1:5000 (ab40793, abcam) and rabbit anti-Akt1 at 1:1000 (9272, Cell Signaling). Secondary horseradish peroxidase-coupled antibodies (rabbit, GE Healthcare) were used at 1:20000. Signals were revealed using an enhanced chemiluminescence reagent (SuperSignal West Pico chemiluminescent substrate, Pierce-Thermo Scientific) by exposure to autoradiography film.

4.6 | Quantitative RT-qPCR

Fat bodies from 4- to 5-day-old virgin female flies were dissected in PBS. Five or more dissected abdomens were lysed in 600 μL Trizol (Invitrogen) and RNA was isolated following the manufacturer's instructions. Reverse transcription was performed using RevertAid reverse transcriptase (Fermentas). We used a CFX thermal cycler (Bio-Rad) and KAPA SYBR FAST qPCR kit (Kapa Biosystems). Expression levels were normalized to the expression levels of *gapdh*. All primer sequences are available upon request. Six independent experiments with more than five abdomens per group were performed. To allow for comparison between experiments, the relative expression levels of in *miR-33* expressing flies were normalized against the expression levels of control flies.

4.7 | Cuticle imaging and quantification of pigmentation

Adult flies were aged for 3 to 5 days post-eclosion and placed in 10% glycerol/95% ethanol for 24 hours prior to dissection. Dorsal abdominal cuticles were dissected in Euparal mounting medium (BioQuip) and imaged with a Leica S8APO dissecting scope and Leica DFC295 camera. Ten cuticles were imaged per genotype and all settings and lighting were kept constant between experimental and control samples. NIH ImageJ was used to quantify the percent of each tergite that was pigmented. For flies using the pnr-*GAL4* driver and their controls, only the percent pigmentation within the approximate pnr expression domain was quantified.

4.8 | Stalk cell and germ cell quantification

Female adult *miR-33* mutant flies and controls were collected within 1 day of eclosion and incubated with males for 3 days in the presence of active dry yeast to stimulate oogenesis. For expression of a *miR-33* sponge specifically in the stalk cells, the 109-53-*GAL4* driver was used. For overexpression of *miR-33*, the 109-53-*GAL4* driver was used in conjunction with a tub-*GAL80^{ts}* transgene, to spatially limit expression of *miR-33* to the stalk cells and temporally limit expression to adulthood, respectively. To control GAL80^{ts} activity, we reared flies at 18°C and later transferred adult females to 29°C for at least 5 days in the presence of males and active dry yeast to stimulate oogenesis. Ovaries were then dissected and stained for Lamin C (1:200, DSHB LC28.26) to visualize interfollicular stalk cells and 1 μg/mL Hoescht to visualize nuclei.[40] Alexa Fluor 568 goat anti-mouse was used as the secondary antibody (Life Technologies). Ovaries were mounted on slides with Vectashield Antifade (Vector Labs). Confocal microscopy was used to visualize ovary abnormalities by scanning from the top to the bottom of the focal plane to detect all stalk cells. Egg chamber stages were identified visually using guidelines outlined by Jia et al.[60] Stalk cells were counted after Stage 2, Stage 3/4, Stage 5/6, and Stage 7/8 egg chambers, as described in Borensztejn et al.[40] Stalk cells posterior to a specific

egg chamber stage were attributed to that egg chamber. Slides were visually scanned for egg chambers; ovarioles were randomly chosen for stalk cell counting. Ovarioles that were twisted or severely overlapping, making stalk cell counting difficult, were not counted.

Abnormal germ cell number was assessed by identifying egg chambers with normal germ cell number (16), greater than 16, less than 16, or with split cysts (eg, g chambers splitting the 16 germ cells). If all egg chambers within an ovariole had normal germ cell numbers, they were counted as "normal." If any egg chamber within an ovariole was abnormal, this was counted in one of the three abnormal categories (greater than 16, less than 16, or split cysts). No ovariole ever contained egg chambers with two different abnormalities.

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AUTHOR CONTRIBUTIONS

Laure-Alix Clerbaux: Conceptualization; formal analysis; investigation; methodology; writing-original draft; writing-review & editing. **Hayley Schultz:** Conceptualization; formal analysis; investigation; methodology; writing-original draft; writing-review & editing. **Samara Roman-Holba:** Conceptualization; formal analysis; investigation; writing-review & editing. **Dan Fu Ruan:** Formal analysis; investigation; methodology; writing-review & editing. **Ronald Yu:** Formal analysis; investigation; methodology; writing-review & editing. **Abigail Lamb:** Formal analysis; investigation; methodology; writing-review & editing. **Guido Bommer:** Conceptualization; funding acquisition; project administration; supervision; writing-review & editing. **Jennifer Kennell:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing-original draft; writing-review & editing.

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