Hedgehog Signaling Strength Is Orchestrated by the mir-310 Cluster of MicroRNAs in Response to Diet

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ABSTRACT Since the discovery of microRNAs (miRNAs) only two decades ago, they have emerged as an essential component of the gene regulatory machinery. miRNAs have seemingly paradoxical features: a single miRNA is able to simultaneously target hundreds of genes, while its presence is mostly dispensable for animal viability under normal conditions. It is known that miRNAs act as stress response factors; however, it remains challenging to determine their relevant targets and the conditions under which they function. To address this challenge, we propose a new workflow for miRNA function analysis, by which we found that the evolutionarily young miRNA family, the mir-310s (mir-310/mir-311/mir-312/mir-313), are important regulators of Drosophila metabolic status. mir-310s-deficient animals have an abnormal diet-dependent expression profile for numerous diet-sensitive components, accumulate fats, and show various physiological defects. We found that the mir-310s simultaneously repress the production of several regulatory factors (Rab23, DHR96, and Ttk) of the evolutionarily conserved Hedgehog (Hh) pathway to sharpen dietary response. As the mir-310s expression is highly dynamic and nutrition sensitive, this signal relay model helps to explain the molecular mechanism governing quick and robust Hh signaling responses to nutritional changes. Additionally, we discovered a new component of the Hh signaling pathway in Drosophila, Rab23, which cell autonomously regulates Hh ligand trafficking in the germline stem cell niche. How organisms adjust to dietary fluctuations to sustain healthy homeostasis is an intriguing research topic. These data are the first to report that miRNAs can act as executors that transduce nutritional signals to an essential signaling pathway. This suggests miRNAs as plausible therapeutic agents that can be used in combination with low calorie and cholesterol diets to manage quick and precise tissue-specific responses to nutritional changes.

KEYWORDS Drosophila; oogenesis; follicle stem cell; Hedgehog signaling; miRNA; the mir-310s; Rab23; dietary restriction; metabolic stress; Hh ligand

ORGANISMS are constantly subjected to changes in nutrient availability and composition, which depend on quantity and quality of consumed food. Currently, there is a considerable amount of data regarding the cellular metabolic processes and signaling pathways involved in metabolism regulation; however, we know little about the mechanisms that efficiently readjust these pathways in response to ever-changing dietary fluctuations. MicroRNAs (miRNAs) are great candidates for such regulation due to their unique features: miRNA expression is extremely dynamic; one miRNA can regulate hundreds of different targets; and more than one miRNA may coordinately regulate a single target. This presents a great number of combinatorial possibilities, which allows for greater precision in regulation of gene expression.

miRNAs have been shown to be involved in virtually all studied biological processes, including regulation of cellular metabolism and organismal homeostasis (Xu et al. 2003; Teleman et al. 2006; Barrio et al. 2014), development of metabolic disorders, and the highly energy-demanding process of carcinogenesis (Bhattacharyya et al. 2006; Leung and Sharp 2010; Ross and Davis 2011). However, it remains extremely difficult to decipher specific in vivo requirements for each miRNA due to the facts that their mutant phenotypes are very subtle (Lai 2015), and most miRNA mutants are
viable, fertile, and apparently normal in well-controlled lab conditions. Furthermore, correlating causal targets to miRNA phenotypes remains the key challenge. Even though multiple algorithms and databases predicting miRNA–messenger RNA (mRNA) interactions based on sequence and physical-chemistry properties exist, they have large numbers of false positives and currently only very few interactions have been experimentally validated. It has been shown that dietary modulations modify miRNA expression profiles, but to date there is a paucity of in vivo functional studies that aim to decipher the complex networks involving nutrition-dependent miRNAs and their targets. Such studies may offer new concepts for preventive and therapeutic strategies for metabolic disorders, including obesity and diabetes.

Since the dietary requirements for major nutrients (sugars, fats, and amino acids) appear to be universal and the signaling pathways involved in the basic logic of nutrient signaling are conserved, studies in model organisms have proven to be beneficial for the understanding of metabolic stress. In Drosophila, similarly to vertebrates, steroids, insulin, and TOR signaling play a critical role in regulation of nutritional responses, suggesting that Drosophila can be used as a relevant model to study nutritional stress (Drummond-Barbosa and Spradling 2001; Konig et al. 2011; Wei and Lilly 2014). Particularly, the Drosophila ovarian germline stem cell community is a very attractive model to study how adult stem cell self-renewal and differentiation is coordinated with organismal metabolism. In the Drosophila germarium, there are two stem cell types of extremely different origin: the germline stem cells (GSCs) and the somatic follicle stem cells (FSCs). These stem cells also have very distinctive stem cell niche types: the stationary, cell–cell adhesion-dependent GSC niche and the dynamic, cell–matrix adhesion-dependent FSC niche (Song and Xie 2002; Nystul and Spradling 2007; Morrison and Spradling 2008). Interestingly, the GSC niche not only controls GSC maintenance, but also has a distant influence on FSC division and differentiation. The FSC gives rise to somatic ovarian cells that come in different types: the follicular epithelium, stalk, polar, and border cells, all of which protect and assist the germline, ensuring sufficient egg differentiation. Therefore, for proper oogenesis progression, it is extremely important that GSC and FSC divisions and the differentiation of their progeny are synchronized (Gilboa and Lehmann 2006; Chang et al. 2013; Konig and Shcherbata 2015). Dependent on nutrient availability, insulin ligands are produced in the brain to activate insulin signaling in the GSCs to cell-autonomously control their division rate; in contrast, the Hh ligand is locally produced by the GSC niche, it travels three to five cell diameters to the posteriorly located FSCs to stimulate their proliferation (Forbes et al. 1996a; Drummond-Barbosa and Spradling 2001; Zhang and Kalderon 2001; O’Reilly et al. 2008; Rojas-Rios et al. 2012).

Importantly, Hh signaling is highly dependent on the diet, because its multiple components are regulated by cholesterol and lipid levels (Panakova et al. 2005; Sieber and Thummel 2012; Hartman et al. 2013). Upon dietary restriction, an organism has to quickly change its cellular metabolism and adapt to unfavorable conditions; however, it is very unlikely that levels of cholesterol and lipids would drop instantly (Efeyan et al. 2015), resulting in sufficient downregulation of Hh signaling. This highlights the importance of the existence of other levels of regulation to ensure the quick and robust response of Hh to dietary changes. While downstream Hh effectors have been well studied in different systems, the upstream regulators of Hh signaling and their roles in energy homeostasis are yet to be revealed. Our data for the first time demonstrate that Hh signaling strength upon nutritional fluctuations can be modulated by miRNAs.

Here we used a new workflow allowing for effective identification of miRNA-regulated processes and relevant targets. First, we applied quantitative proteomic analysis of miRNA mutants to identify the major biological processes affected by miRNA loss. Second, tissue-specific dissection of miRNA mutants was performed to identify the most prominent phenotypes caused by miRNA insufficiency. Third, based on the vast amount of previously published data, we compared these phenotypes to the key phenotypes associated with major signaling pathways. Fourth, we used several databases (Enright et al. 2003; Kheradpour et al. 2007; Betel et al. 2008) to predict potential miRNA targets, among which several genes relevant to the identified signaling pathway were selected and further confirmed using in vitro and in vivo assays. Finally, genetic analyses and rescue experiments under normal and defined stress conditions were performed to validate miRNA roles in certain biological processes.

Using this paradigm, we found that in Drosophila during adulthood, the mir-310s orchestra Hh signaling strength in accordance with nutritional status. We identified three new mir-310s targets, Rab23, DHR96, and ttk, all of which are involved in Hh pathway regulation. By simultaneous targeting of multiple regulators of the pathway, the mir-310s safeguard a quick and robust response of Hh signaling to dietary fluctuations. Additionally, we discovered a molecular function for the membrane trafficking protein Rab23 in the process of Hh ligand intracellular transport and secretion in the stem cell niche. Plausibly, Hh signaling management by the mir-310s is just one example of many diet-dependent processes regulated by these miRNAs. Our proteomic data, generated by SILAC labeling accompanied by mass spectrometry analysis, revealed that multiple critical metabolism-related genes are deregulated due to the mir-310s deficiency under normal and dietary restrictive conditions, suggesting that in general, the molecular function of these miRNAs is management of organismal homeostasis upon dietary fluctuations.

**Materials and Methods**

**Fly stocks**

All fly stocks were maintained and crosses were set up on standard food with yeast, cornmeal, and agar at 25°C, constant humidity, and a 12-hr light–dark cycle. The nutrient restriction experiments were done using 2% agar-agar (Serva), 25% apple...
juice, and 2.5% sugar medium. The nutrient-starved flies were fed this medium plain, whereas the well-fed flies were given additional fresh yeast paste made of dry yeast and 5% propionic acid (≈50% w/v). Food vials of both conditions were refreshed every 2 days. The following fly stocks were used: Oregon-R-C and w¹¹¹8 as controls; mir-310s deletion lines KT40 (Tsurudome et al. 2010), w⁰; Df(2R)mir-310s-311-312-313 P(neuroRT42D)/CyO, P(GAL4-twist.G)2.2, P(UAS-2xEGFP)AHH2.2 (no. 58923 Bloomington Drosophila Stock Center, BDSC), and the deficiency line w¹¹¹8; Df(2R)Exel6070, P[w¹¹¹8-mC]=XP-U]Exel6070/CyO (no. 7552 BDSC) as mutant alleles; mir-310s-Gal4 (P(GawB)NP425S from Drosophila Genomics and Genetic Resources, Kyoto) (Yatsenko et al. 2014), UAS-mCD8::GFP, UAS-nLacZ line (gift from Frank Hirth) for expression analyses; tub-Gal80ts (no. 28025 BDSC), UAS-hh (gift from Christian Bökel), UAS-Rab23 RNAi (y¹¹¹8; P(y¹¹¹8-t7.7) v¹¹¹8-t1.8]=TRiP.JF02859)attP2 (no. 28025 BDSC), UAS-hh RNAi (Sahai-Hernandez and Nystul 2013), and a mir-310s rescue line (w⁺; Soc/CyO; attB2 mir-310s res long 2/TM6B) carrying a large genomic region encompassing the mir-310s as a transgene in the 3rd chromosome (gift from Eric Lai) for rescue experiments. To generate the UAS-Rab23 line, we cloned Rab23 cDNA into the UAS vector (gift from Alf Herzig). Cloning was performed by standard cloning techniques, digesting the Rab23 cDNA vector (RH23273 clone from Drosophila Genomics Resource Center) and the UAS vector with EcoRI and KpnI restriction enzymes. The microinjection and recovery of the transgenic flies was done by Bestgene. The site-specific integration on the 3rd chromosome (76A2 site) was achieved by the att sites in the UAS-Rab23 plasmid and PhiC31 into the PBac[yellow+] attP-9A]VK00013 strain. Rab23::YFP::4xmyc line (also referred as Rab23::YFP in the text) was generated by ends-in homologous recombination, and the initial genomic duplication was resolved using the I-Cre system. The size of the homologous sequence 5’ from the YFP start codon is 4045 bp, and the size of the homologous sequence 3’ from Myc tag DNA fragment is 3601 bp. The donor construct was verified by sequencing. Recombination events were verified by PCR. In our analyses, homozygous flies bearing endogenously tagged Rab23 copies were used.

For SILAC analysis, qRT-PCR (list of primers, Table S11), immunohistochemistry, luciferase assay, coupled colorimetric assay (CCA), and communoprecipitation, refer to File S1.

Results

mir-310s loss of function causes defects in energy metabolism and deregulation of nutritional homeostasis-associated genes

In our previously performed screen for stress-dependent miRNAs (Marrone et al. 2012), we found that the miRNAs from the newly evolved mir-310s family are differentially expressed under stress and disease conditions. Therefore, we aimed to decipher the potential role for these miRNAs in maintenance of a healthy physiological state. To begin with, we studied global changes in protein expression caused by mir-310s deficiency. The quantitative SILAC proteomics data of miRNA mutant flies were generated for the first time using previously described (Sury et al. 2010) mass spectrometry of heavy isotope-labeled Drosophila. This analysis resulted in a sizeable list of proteins with altered expression levels caused by mir-310s deficiency. Since miRNAs are generally identified as fine tuners of gene expression, we considered proteins with a moderate (≥30%) relative increase or decrease with a P-value of 0.1 for filtering for the significant data (Supporting Information, Table S1), which resulted in the identification of 264 proteins that were up- or downregulated in mir-310s mutants, among which 24 are predicted mir-310s direct targets (Table S1, bold boxes).

Next, using the STRING database (Franceschini et al. 2013), we created functional association networks of deregulated genes; then, we grouped these genes into functional groups according to their gene ontology (GO) terms from the UniProt database (UniProt Consortium 2014). This analysis revealed distinct functional groups: lipid and energy metabolism, protein homeostasis, nucleotide synthesis, mitochondria, muscle and neural development/function, cuticle formation, and others (Figure 1A). Furthermore, 20% of the altered genes were reported to be lipid droplet associated (Kuhnlein 2011).

Importantly, the common denominator of these affected gene functions was their involvement in energy metabolism and homeostasis, suggesting that the mir-310s are involved in regulation of these processes, which can be achieved directly by the mir-310s regulation of their target genes and indirectly by secondary effects of their targets. It is important to stress that due to the current limitations of quantitative mass spectrometry analyses, only 30% of all predicted Drosophila proteins could be identified in this study, which is comparable to previously described SILAC proteomic data (Sury et al. 2010). Detected proteins mainly represent the most highly expressed, but not regulatory proteins or transcription factors that are known to efficiently operate even in very low quantities. Despite the limitations of this analysis in identifying direct miRNA targets that belong to these functional groups, it allowed for meaningful identification of the processes regulated by the miRNAs.

Since our proteomics data indicated that the mir-310s could be associated with maintenance of metabolism and energy homeostasis, we compared this dataset with genes previously analyzed as starvation-sensitive by transcriptome analysis (Farhadian et al. 2012) and found 31 genes in common. Next, we measured mRNA expression levels of these genes by qRT-PCR in wild-type and mir-310s mutant animals under well-fed and nutrient-restricted conditions (Figure 1B; Table S2). We used two diets: well-fed (sugars + yeast paste) and starved/protein restricted (just sugars, no yeast paste). As has been reported by pioneering studies and recent efforts, the Drosophila life cycle (development and adult homeostasis) greatly depends on the nutritional input from the yeast source, which can be reconstituted by addition of amino acids, cholesterol, nucleic acids, folic acid, inositol, biotin, riboflavin, nicotinic acid, pyridoxine hydrochloride, calcium
pantothenate, thiamine, choline chloride, ergosterol, and metal ions (Piper et al. 2014). Our starvation conditions supply only simple sugars and lack these essential components needed for optimal homeostasis. In agreement with the proteomic data, most of these genes were aberrantly expressed in mir-310s mutants. In addition, starvation induced uncoordinated alterations in the gene expression profiles, consistent with a suggested role for mir-310s in dietary response (Figure 1, A and B). For instance, one of the genes, Larval serum protein 1 β (Lsp1β), was found to be 10-fold...
higher in mir-310s mutants under well-fed conditions in comparison to controls. Nutrient restriction caused a sharp decrease (>30-fold) of the Lsp1beta transcript levels in control flies, while almost no change was detected in mir-310s mutant flies (only a one third-fold decrease). The transcript levels of another gene, Larval serum protein 2 (Lsp2) were downregulated close to zero upon nutrient restriction in control flies; however, in mir-310s mutants, Lsp2 levels were only slightly decreased. As a result, Lsp2 mRNA levels in nutrient-restricted mir-310s mutants were ~40-fold higher in comparison to those in controls. In general, most of the nutrition-dependent genes that were analyzed showed atypical alterations in their expression levels in mir-310s when compared to wild-type flies under both normal and restrictive conditions, showing a role for the mir-310s in nutritional homeostasis and response to starvation.

Defects caused by mir-310s deficiency depend on nutrition

In correlation with the abnormal expression of the energy- and lipid metabolism-related genes, the analysis of mir-310s-deficient flies revealed several gross morphological and physiological phenotypes that are known to be related to nutrient availability. One of the most prominent phenotypes detected upon dissection of mir-310s mutants was the enlarged food storage organ or crop (Figure S1, A and A’), the size of which is highly diet dependent and is capable of expansion after starvation and refeeding (Lemaître and Miguel-Aliaga 2013). It is also known that the enlarged crop is a persisting signature of poststarvation response, since females switched from nutrient-poor to nutrient-rich food consume more food (Edgecomb et al. 1994; Al-Anzi et al. 2010). We found that even under normal feeding conditions, average crop size of mir-310s females was 30% larger than crops of wild-type females of comparable size (Figure S1A’). This suggests that due to their abnormal metabolism, mir-310s females exhibit a phenotype consistent with the physiology elicited during poststarvation.

Interestingly, studies on the physiology of starvation-selected flies demonstrate that their entire life history is disturbed; as adults, these animals contain more lipids, but at the cost of reduced fecundity (Masek et al. 2014). To determine whether mir-310s flies exhibit these phenotypes, first we evaluated the fat storage characteristics of mutant females using a colorimetric assay. Under well-fed conditions, the total body fat content of mir-310s females was ~2-fold lower than that of controls (Figure S1B). Consistent with previously reported data (Musselman et al. 2011), 10 days of protein starvation resulted in a 1.3-fold increase in the total body fat content in controls. However, upon the same restriction, mir-310s females accumulated dramatically higher amounts of lipids, 2.5- and 4-fold increases in comparison to the starved and well-fed controls, respectively (Figure S1, B and B’).

It is well known that the nutrient-sensitive and energy-demanding egg production process is stopped due to nutrient deficit (Drummond Barbosa and Spradling 2001). In mir-310s females, the cessation of egg production is delayed compared to wild type in response to starvation (Figure S1C). However, even on a normal diet, mir-310s females laid ~2.5-fold fewer eggs (Figure S1, D–E). If egg-laying ability is a direct readout of metabolic status, these results imply that mir-310s-deficient females in general have deficient energy resources and in addition, they cannot properly respond to dietary restriction. Taken together, the proteomic and qRT-PCR expression assays (Figure 1) in combination with the physiological defects caused by mir-310s loss, which include increased crop size and reduced egg production under normal diet and dramatic fat accumulation under starvation (Figure S1, Table S3), confirm that the mir-310s are essential factors in regulation of energy metabolism in various physiological and cellular elements of the whole organism.

The mir-310s function in the ovarian soma

Next, we aimed to dissect the mir-310s function at the cellular level and identify their direct targets involved in starvation response. Therefore, we focused on oogenesis, which is one of the best-studied nutrition-dependent processes. Drosophila oogenesis takes place in the ovaries, which are paired organs consisting of individual ovariololes—the egg production units made of progressively developing egg chambers. While developing egg chambers move toward the posterior and develop into mature eggs, they stay attached to the neighboring egg chambers by small groups of cells forming stalks (Figure 2A). Each egg chamber is surrounded by a monolayer of epithelium composed of follicle cells, and specialized polar cells are specified at each end of the egg chamber.

To identify the possible involvement of the mir-310s in oogenesis, we analyzed their expression pattern, which was visualized by nuclear β-gal and membrane-bound GFP driven by mir-310s–Gal4. Expression of reporters was detected in subsets of different somatic cell types, and their expression levels were fluctuating (Figure 2A). For example, some of the stalk and follicular epithelium cells were expressing nuclear β-gal and/or membrane GFP, but some were not (Figure 2, B and C). Since β-gal and GFP proteins have different turnover rates (Timmons et al. 1997), we conclude that expression of the mir-310s in the ovarian soma is dynamic. Similarly, a dynamic mir-310s expression pattern was observed in the brain, where the precision of these miRNAs’ expression is achieved via the perceptive-executive mechanism orchestrated by their target (Yatsenko et al. 2014).

Upon in-depth examination of mir-310s mutant ovaries in well-fed conditions, we identified several phenotypes in the ovarian soma, which could be categorized into three distinct groups. First, supernumerary stalk cells accumulated between egg chambers: in control ovarioles, up to eight stalk cells properly line up between adjacent egg chambers, while in mir-310s ovarioles, excessive numbers of disorganized cells at the stalk region formed a multilayered epithelium (Figure 2D). Second, the follicular epithelium cells surrounding egg chambers of different stages were distorted in shape and had irregular cellular polarity, assembling random multilayered
patches (Figure 2D). Third, abnormally encapsulated egg chambers, easily identifiable by the aberrant numbers of polyploid nurse cells, appeared (Figure 2D). These defects were very similar to the previously described ovarian phenotypes caused by defective Hh signaling (Figure 2E). Hh signaling is important for cell fate establishment of all ovarian somatic cell types (Forbes et al. 1996a,b; Tworoger et al. 1999; Besse et al. 2002; Chang et al. 2013) and its ligand is significantly upregulated upon starvation. Whole ovary extracts from 7-day-starved females show an ~1.5-fold increase in miRNA levels compared to well-fed controls (Table S5). The bar graph indicates AVE ± SEM. Significances were calculated with two-tailed Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005. At least three biological replicates per genotype and condition were analyzed. (I) Upon 7 days of nutritional restriction, the number of mir-310s expressing CpCs significantly increases as visualized by anti-GFP staining (mir-310s-Gα4lf/+; UAS-mCD8::GFP, UAS-nlacZ2/+). The bar graph indicates AVE ± SEM values are reported from the measurements done from 20 germaria (4.1 ± 0.34 well-fed, 5.6 ± 0.42 starved, statistical significance is calculated using Mann–Whitney U-test and Z-statistic, P = 0.0078). In A–G, anterior is to the left. B and C represent single optical sections. A, D, E, and G represent maximum intensity projections of confocal Z-stacks. Bars, 20 μm in A, D, and E and 5 μm in B, C, and G.
produced by the terminal filament and cap cells (TFs and CpCs) forming the stem cell niche, and also escort cells (ECs) forming the germline differentiation niche (Rojas-Ríos et al. 2012). The dynamic mir-310s expression was detected in all of these niche cell types (Figure 2, F and G). Importantly, this expression appeared to be diet-sensitive; upon starvation, mir-310s expression levels were upregulated (Figure 2H), and the number of mir-310s-expressing GSC niche cells (CpCs and TFs) was significantly increased (Figure 2I). These results suggest that the mir-310s have a cell-autonomous role in the stem cell niche during dietary changes.

The analysis of the mir-310s expression pattern revealed that the mir-310s are dynamically expressed in the Hh signal-sending cells (TFs, CpCs, and ECs in the gerarium) as well as in the Hh signal-receiving cells (the stalk and follicular epithelium cells in the developing egg chambers) (Figure 2, A–C and G). These results, combined with the similarities of the observed mir-310s loss- and Hh gain-of-function mutant phenotypes (Figure 2, D and E), led us to hypothesize that the mir-310s regulate Hh signaling via targeting one or multiple components of this pathway.

mir-310s target three genes associated with the Hedgehog signaling pathway

To confirm this hypothesis and define the molecular mechanism responsible for mir-310s ovarian phenotypes, we acquired a list of in silico-predicted mir-310s targets using several miRNA target search databases (Enright et al. 2003; Kheradpour et al. 2007; Betel et al. 2008) and selected among the putative mir-310s targets all known or predicted Hh pathway elements and their interaction partners. The mir-310s are recently evolved miRNAs, which have highly evolutionarily conserved seed sequences (Figure 3A). As predicted by different algorithms, the mir-310s have 350–450 putative targets, among which only three [Rab23, tramtrack (ttk), and Hormone receptor-like in 96 (DHR96)] have been associated with Hh signaling.

To verify that the mir-310s indeed target these three genes, we performed a Drosophila S2 cell-based luciferase reporter assay, which depends on the readout from a reporter plasmid with a luciferase gene containing the 3’UTR of the gene of interest with the predicted miRNA target site. The luciferase assay showed that in vitro, the mir-310s could target the Rab23, DHR96, and ttk transcripts via their 3’UTRs (Figure 3B; Table S4).

Next, we tested in vivo the responsiveness of these three putative target genes to the mir-310s as well as to nutrient restriction. We found that the expression of all three genes is nutrition dependent, showing significant reduction under starvation conditions. In mir-310s mutants, Rab23 and DHR96 levels were significantly upregulated (>1.5-fold; Figure 3C), and their expression levels were not as efficiently reduced under starvation. In contrast, ttk mRNA expression levels were similar to controls in mir-310s-deficient flies under well-fed conditions. ttk expression was controlled by the mir-310s only under nutritional stress, where mir-310s mutants had 1.5-fold higher ttk mRNA levels when compared to controls (Figure 3C and Table S5). These data demonstrate that the mir-310s act to fine tune the expression of the nutrition-dependent genes Rab23, DHR96, and ttk; furthermore, the mir-310s regulate ttk only upon dietary restriction.

The above results confirm that the mir-310s are important regulators of at least three components associated with Hh signaling (Figure 3D). DHR96 encodes a cholesterol receptor responsible for sensing the nutritional status of the cell environment (Horner et al. 2009; Bujold et al. 2010; Sieber and Thummler 2012) and promoting Hh ligand release upon dietary cholesterol intake (Hartman et al. 2013). ttk encodes a transcription factor that acts as a controller of the cell cycle switch during midoogenesis through regulation of Hh target gene expression (Sun and Deng 2007). Rab23 encodes a membrane organization and trafficking Rab GTPase (Zerial and McBride 2001; Zhang et al. 2007; Chan et al. 2011). Mouse Rab23 was shown to act as a negative regulator of the Sonic Hh signaling pathway in signal-receiving cells during embryonic neural patterning (Eggenschwiler et al. 2001). However, Drosophila Rab23 is known not to function in Hh signaling through the same mechanism [at least in the process of wing development (Pataki et al. 2010)], and its role in Hh signaling has not been confirmed.

The mir-310s and Rab23 regulate Hh ligand release

As the involvement of Rab23 in the Hh pathway remains an open question in Drosophila (Zhang et al. 2007; Pataki et al. 2010), we decided to further focus on the mir-310s–Rab23 interaction. First, we analyzed the spatial distribution of Rab23 protein using a Rab23::YFP line generated via homologous recombination (see Materials and Methods). Similarly to the mir-310s, the endogenous Rab23 protein was detected in the germline stem cell niche (TFs and CpCs) and in the differentiation niche (ECs), and this expression was dynamic: some of the niche cells were Rab23 negative and the others could be classified as high or low Rab23-expressing cells (Figure 2G, Figure 4, A–D). Upon close examination, we found significantly more Rab23-positive CpCs in mir-310s mutant germaria in comparison to controls (Figure 4E). Moreover, upon starvation, the number of cells with high Rab23 levels was significantly increased in mir-310s mutants (Figure 4E; Table S6).

In wild type, Hh is produced in the stem cell niche and travels into the posterior compartment to activate FSC division. We observed that the elevated levels of Rab23 in mir-310s mutants in different conditions coincided with higher levels and a broader expression pattern of the Hh ligand, as detected by an anti-Hh antibody (Figure 4, A–D). To confirm the roles of Rab23 and the mir-310s in the dispersion of the Hh ligand, we calculated the number of Hh protein speckles in the gerarium (Figure 4, A–D, F, and G). Indeed, mir-310s loss and Rab23 overexpression in the stem cell niche both resulted in significantly higher numbers of Hh speckles distributed throughout the whole gerarium (Figure 4, C and F, red line). Moreover, although starvation results in the restriction of Upon Diet, miRNAs Modulate Hh Signaling

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Hh ligand to the anterior part of the germarium (Hartman et al. 2013) (Figure 4B, red line), in the starved mir-310s loss-of-function and Rab23 overexpressing germaria, this spatial restriction was less pronounced (Figure 4, D–H, red lines; Table S7). These results confirm a role for Rab23 in the cell-autonomous positive regulation of Hh release and suggest that the effect of mir-310s deficiency on Hh ligand distribution occurs via Rab23.

Next, we tested if starvation-mediated regulation of hh expression occurs at the transcriptional level. However, expression of a hh-lacZ reporter transgene in the stem cell and differentiation niches did not change upon starvation (compare Figure 4I and 4J, arrows and arrowheads, respectively). These data indicate that upon dietary restriction, Hh is not regulated at the transcriptional level; on the contrary, the mir-310s and Rab23 play a role in Hh spatial distribution.

Next, we aimed to understand how Rab23 is involved in regulation of the Hh ligand. Analysis of Rab23 and Hh protein expression in CpCs revealed dynamic expression patterns such that some cells coexpressed both proteins and others were positive for either Hh or Rab23 (Figure 4, A–D). At the subcellular level, the proteins formed puncta, some of which could intrinsically regulate these targets in both the Hh signal-sending and Hh signal-receiving cells of the ovarian soma in response to nutrient availability. For B and C, bar graphs indicate AVE ± SEM. Significances were calculated with two-tailed Student’s t-test. *P < 0.05, **P < 0.005, ***P < 0.0005.
Figure 4 Rab23 is targeted by the mir-310s, controlling Hh ligand availability. (A–D) The mir-310s negatively regulate Rab23 expression. Rab23 has a stronger and more widespread expression pattern in mir-310s mutant (C, KT40/KT40; Rab23::YFP::4xmyc) compared to control germaria (A, w1118; Rab23::YFP::4xmyc). As a result of 7-day starvation, in controls, Rab23 has more widespread staining (B), which is more obvious in mir-310s mutants (D).

The Hh ligand is produced by CpCs in the niche (outlined in white) and is visualized by anti-Hh antibody (red). Hh protein is detected along the length of the germarium under normal conditions (A). Upon starvation, Hh speckles are confined at the anterior half of the germarium (B, red line), while in the mir-310s mutants (D).
contained Hh or Rab23 only (Figure 4K, red and green arrows, respectively) and some had both proteins colocalized (Figure 4K, yellow arrows). In general, Rab proteins are vesicle-tethering proteins, regulating intracellular trafficking (Zerial and McBride 2001). Therefore, we hypothesized that Rab23 is involved in transport and trafficking of Hh-loaded vesicles in the GSC niche cells. To test this idea, we performed a communoprecipitation using Rab23::YFP::4xmyc flies to identify Rab23 interaction partners. Subsequently, mass spectrometry analysis followed by GO term analysis of identified proteins (UniProt Consortium 2014) and evaluation of functional association networks (Franceschini et al. 2013) revealed a group of 12 proteins as components of COPI-coated vesicle machinery among a larger number of other identified proteins (Figure 4L; Table S8). Importantly, this implicates Rab23 as a novel regulator of precisely controlled Hh ligand secretion in Drosophila. In summary, our results suggest a model in which mir-310s act in the stem cell niche to repress expression of Rab23, which is involved in intracellular vesicle trafficking and release of the Hh ligand in COPI vesicles.

**mir-310s moderate ovarian Hh signaling via downregulation of the positive regulator Rab23**

If our model is correct, then the diet-dependent mir-310s–Rab23–Hh trafficking cascade should have a direct effect on Hh signaling strength, and manipulation of the levels of these components may allow the rescue of phenotypes associated with abnormal Hh signaling. Therefore, we performed such an epistasis analysis, quantifying several ovarian phenotypes previously described as Hh signaling defects (Forbes et al. 1996a,b). First, we analyzed the posterior gerarium architecture at the intersection of regions 2a and 2b (Figure 2F). In controls, ~75% of germaria had germline cysts fully encapsulated by the follicular epithelial cell precursors (Figure 5A, marked by FasIII, arrow), while ectopic Hh expression in the GSC niche results in the accumulation of germ cells in germarial region 2b (Figure 5, A, A’, and B). Next we analyzed mir-310s loss-of-function and Rab23 niche-specific gain-of-function phenotypes and compared their frequencies to those caused by Hh overexpression. Only 20% of mir-310s mutant germaria had this region properly structured (Figure 5, A and B). This phenotype is mir-310s specific, since it was observed in different mir-310s mutant allelic combinations and could be fully rescued by the introduction of a mir-310s genomic fragment (Figure 5, A”” and B; Table S9). Similar frequencies of disorganization were observed due to Rab23 overexpression (Figure 5, A’’’ and B). If this defect is caused by the increased levels of the Hh ligand, trafficking and release of which depend on Rab23, which is in turn negatively regulated by the mir-310s, then downregulation of Hh or Rab23 should alleviate mir-310s-deficient phenotypes in the gerarium. Indeed, this germlarial defect was significantly rescued by reducing Rab23 or Hh levels via RNAi in a mir-310s mutant background (Figure 5B; Table S9).

Second, we analyzed the germline pinching-off defects. Abnormal cyst encapsulation in the gerarium coupled with defective epithelial cell fate determination results in the appearance of egg chambers containing atypical numbers of germline cells (Figure 2, D and E; Figure 5, C–E). This phenotype was detected in ~40% of mir-310s-deficient and Rab23-overexpressing ovarioles and was even more pronounced (~90%) in the ovarioles with Hh overexpression (Figure 5, C–F). Importantly, the introduction of a mir-310s genomic fragment or reduction of Hh or Rab23 in mir-310s mutants fully rescued this phenotype, demonstrating that Rab23 and Hh act downstream of the mir-310s in this process (Figure 5F; Table S9).

Third, we analyzed the state of stalk cell specification. Increased Hh levels cause abnormal differentiation of the stalk cells, resulting in the accumulation of excessive precursor stage-like cells (Tworoger et al. 1999). A total of 70% of all ovarioles contained multilayered stalks in mir-310s loss of function (Figure 5G) and Rab23 gain of function (Figure 5E), and again this phenotype was even stronger in the Hh gain of function (Figure 2E). This phenotype was fully rescued upon the introduction of a mir-310s genomic fragment (Figure 5H) or downregulation of Rab23 or Hh in mir-310s-deficient animals (Figure 5I; Table S9).

Normally, the stalk cells are terminally differentiated epithelial cells that never divide; thus, a multilayered stalk phenotype can result if stalk cell differentiation is delayed
Figure 5 The mir-310s and Rab23 regulate Hh signaling in the ovary. (A and B) Prior to the pinching off of the egg chamber from the gerarium, the germline cysts are encapsulated by follicle cell precursors marked with FasIII, which move toward the interior of the gerarium and envelop the cyst (A, arrowhead) as shown in a control (w1118/Oregon-R-C) gerarium. Hh overexpressing (A', tub-Gal80ts; bab1-Gal4/ UAS-hh at 29°), mir-310s mutant (A'', KT40/Df(6070), and Rab23 overexpressing (A''', bab1- Gal4/UAS-Rab23) germaria have disorganized architecture at the posterior end, with a significantly lower frequency of properly encapsulated cysts than in controls. This phenotype can be rescued by introducing a mir-310s genomic rescue construct in the mir-310s mutant background (A'', KT40/Df(6070), bab1-Gal4/ UAS-hh-RNAi (Table S9)). (C–F) mir-310s deficiency causes the appearance of egg chambers with abnormal sizes and an abnormal number of nurse cells (C). In addition, the follicular epithelium becomes multilayered in irregular patches (arrowhead in C). Similarly, Hh or Rab23 overexpression results in the occurrence of egg chambers with similar defects (D and E). The frequency of this phenotype is comparable for mir-310s mutation and Rab23 overexpression. This phenotype can be rescued by downregulating the Rab23 or Hh levels in a mir-310s mutant background (KT40/Df(6070), bab1-Gal4/UAS-Rab23-RNAi and KT40/Df(6070); bab1-Gal4/UAS-hh-RNAi (Table S9)). (G–I) Loss of the mir-310s results in an excess number of cells accumulating between the egg chambers (arrowhead), forming an overcrowded, multilayered stalk. This phenotype can be rescued by introducing the mir-310s genomic rescue construct in a mir-310s mutant background (H, KT40/Df(6070), bab1-Gal4/UAS-hh-RNAi at 18°) (J, arrowhead). Higher levels of Hh overexpression result in a severe phenotype (depicted in Figure 2E). Rab23 overexpression causes the same stalk defects (J'''). This phenotype can be rescued by introducing the mir-310s genomic rescue construct in the mir-310s mutant background (J''', KT40/Df(6070), bab1-Gal4/UAS-hh-RNAi at 18°) (J, arrowhead). Higher levels of Hh overexpression result in a severe phenotype (depicted in Figure 2E). Rab23 overexpression causes the same stalk defects (J''''). This phenotype can be rescued by introducing the mir-310s genomic rescue construct in the mir-310s mutant background (J''', KT40/Df(6070), bab1-Gal4/UAS-hh-RNAi at 18°) (J, arrowhead). Higher levels of Hh overexpression result in a severe phenotype (depicted in Figure 2E). Rab23 overexpression causes the same stalk defects (J'''''). This phenotype can be rescued by introducing the mir-310s genomic rescue construct in the mir-310s mutant background (J'''', KT40/Df(6070), bab1-Gal4/UAS-hh-RNAi at 18°) (J, arrowhead). Higher levels of Hh overexpression result in a severe phenotype (depicted in Figure 2E). Rab23 overexpression causes the same stalk defects (J''''''). This phenotype can be rescued by introducing the mir-310s genomic rescue construct in the mir-310s mutant background (J''''', KT40/Df(6070), bab1-Gal4/UAS-hh-RNAi at 18°) (J, arrowhead). Higher levels of Hh overexpression result in a severe phenotype (depicted in Figure 2E). Rab23 overexpression causes the same stalk defects (J'''''''). This phenotype can be rescued by introducing the mir-310s genomic rescue construct in the mir-310s mutant background (J''''''', KT40/Df(6070), bab1-Gal4/UAS-hh-RNAi at 18°) (J, arrowhead). Higher levels of Hh overexpression result in a severe phenotype (depicted in Figure 2E). Rab23 overexpression causes the same stalk defects (J''''''''').
and proliferation continues. In this case, these cells would express undifferentiated epithelial precursor cell markers and undergo additional divisions. Therefore we analyzed the expression of a precursor cell marker, FasIII, in stalks between late-stage egg chambers (later than stage 6) that normally no longer express FasIII (Figure 5, J–J999).

Overexpression of the Hh ligand in the GSC niche results in a very dramatic phenotype, in which all the stalk cells were abnormally differentiated (Figure 2E and Figure 5D). Therefore, to obtain stalks with a less severe phenotype more amenable to quantification, we overexpressed Hh in a short pulse during adulthood using the Gal4/Gal80ts system. Hh overexpression in the stem cell niche led to the appearance of stalk cells with persistent FasIII expression between late-stage egg chambers (Figure 5J). Similarly, we found FasIII-positive stalk cells in 50% of the analyzed stalks in mir-310s loss-of-function and Rab23 overexpressing ovarioles (Figure 5, J and J99); Table S9). Importantly, downregulation of Hh signaling, either by mir-310s genomic rescue or by downregulation of Rab23 or Hh ligand expression in the GSC niche, rescued the stalk cell specification phenotype (Figure 5K; Table S9).

Together, these phenotypic analyses indeed show that higher levels of Rab23 in mir-310s mutants phenocopy overactive Hh signaling and confirm that the effect the mir-310s have on Hh signaling is accomplished via their regulation of Rab23. Thus, Hh signaling can be intensified via Rab23-mediated enhancement of Hh ligand trafficking/release and the mir-310s moderate this signaling cascade via the upstream targeting of Rab23.

**Figure 6** The phenotypes caused by mir-310s loss or hh overexpression can be alleviated by dietary restriction. (A–E) hh gain of function causes epithelial defects resulting from somatic cell overproliferation (A, arrows). Upon nutritional restriction for 3 days, the dramatic hh gain-of-function (tub-Gal80⁰⁺; bab1-Gal4/UAS-hh at 29⁰) phenotypes become significantly less penetrant (B and E). Similarly, the appearance of the atypical multilayered epithelium in mir-310s mutant (C, arrows, KT40/Df6070) ovaries is dramatically reduced upon nutritional restriction (C–E) (Table S9). (F–H) Under nutritional stress, on average less than one mitotically active follicle cell (marked by PH3) per stage 2 egg chamber is found in controls (F and H). In the mir-310s mutant, this number is increased (G and H). After nutritional restriction for 7 days, egg production is slowed down, which results in a reduction of follicular epithelial cell proliferation. However, the number of PH3-positive cells is fourfold higher due to mir-310s loss (H). Similarly, upon starvation, overexpression of Rab23 (tub-Gal80⁰; bab1-Gal4/UAS-Rab23) and Hh (tub-Gal80⁰; bab1-Gal4/UAS-hh) (4 days at 29⁰) results in an approximately fivefold higher PH3-positive cell number compared to control (H). The high mitotic activity in mir-310s mutant egg chambers is rescued by an independent genomic mir-310s rescue construct (KT40/KT40; attB2 mir-310s rescue long2/+), or by downregulating the Rab23 (KT40/KT40; bab1-Gal4/UAS-Rab23-RNAi) or Hh levels (KT40/KT40; bab1-Gal4/UAS-hh-RNAi) (Table S10). A–D, F, and G represent single optical sections and anterior is to the left. Bars, 20 μm in A–D and 5 μm in F and G. In H, the bar graph indicates AVE ± SEM. Significances were calculated for E using Pearson’s chi-square test (Table S9) and for H, using Mann–Whitney U-test and Z-statistic. *P < 0.05, **P < 0.005, ***P < 0.0005 (Table S10).
**Hh signaling is regulated by the mir-310s in response to diet**

Since our data show that *mir-310s* mutants show defective metabolic status (Figure S1), and that the *mir-310s* act upstream of Hh signaling via repression of Rab23 (Figure 3C; Figure 4, C–E), we decided to test whether these miRNAs would aid in adjusting Hh signaling efficiency in response to nutritional stress. Remarkably, we observed that the dramatic ovarian phenotypes associated with excessive Hh signaling were radically improved upon dietary restriction (compare Figure 6A and 6B). Similarly, the appearance of mutant phenotypes in *mir-310*so variants was significantly rescued upon starvation (compare Figure 6C and 6D). To quantify the effect of starvation, we focused on the overproliferated stalk phenotype, since it is a hallmark of hyperactive Hh signaling. A total of 100% of Hh-overexpressing egg chambers contained patches of multilayered stalk cells, while upon starvation the frequency of this phenotype was reduced by half; in *mir-310* s, this phenotype was fully rescued by dietary restriction (Figure 6E; Table S9).

It is known (Forbes et al. 1996a,b) that ectopic *hh* expression results in excessive somatic cell proliferation and that stimulated Hh release can induce ligand accumulation on the follicle cells, hence promoting their division even under nutrient-restricted conditions (Hartman et al. 2013). Therefore, we analyzed the number of mitotically active cells among follicular epithelium cells wrapping stage 2 egg chambers using phosphohistone 3 (PH3) as a mitotic marker. Under normal conditions, *mir-310s* mutant, Hh and Rab23 overexpressing egg chambers also have a mild increase (1.2- to 2-fold) in the number of follicle cells in mitosis (Table S10). This tendency became even more pronounced under starvation, as *mir-310s* mutants had almost 4-fold higher numbers of follicle cells in mitosis when compared to controls (Figure 6, F–H). To determine whether this proliferation phenotype is caused by excessive Rab23 levels and, thus, overactive Hh signaling, we overexpressed Rab23 or Hh ligand in Hh-sending cells. As expected, even upon starvation, both of the overexpression experiments resulted in ~5-fold higher numbers of dividing follicle cells in the follicular epithelium, suggesting that Rab23 cell-autonomous involvement in the Hh signaling pathway is diet dependent. Notably, the excessive *mir-310s* follicle cell division phenotype under starvation was significantly rescued by the introduction of the *mir-310s* genomic locus or downregulation of Rab23 or *hh* in a *mir-310s* mutant background (Figure 6H; Table S10). These results show that the abnormal follicle cell proliferation upon dietary restriction is caused by the higher levels of Rab23, and, consequently, overactive Hh signaling as a result of *mir-310s* loss of function. Furthermore, this confirms our hypothesis that the *mir-310s*–Rab23–Hh ligand signaling cascade regulates Hh signaling activity, and this regulation becomes even more prominent in response to dietary fluctuations.

Together, our data show that the miRNAs pathway plays an important role in adjusting the metabolic status of an organism to nutritional signals. In particular, we found that the *mir-310s* are diet-sensitive and that *mir-310s*-deficient flies exhibit severe abnormalities in metabolic homeostasis, including altered gene and protein expression profiles. In addition, multiple diet-sensitive physiological processes, such as crop size, lipid storage, and fecundity are perturbed. Furthermore, we found that the *mir-310s* are capable of targeting at least three genes associated with the Hh signaling pathway, ensuring a robust, fast, and precise response to diet alterations via modulation of this vital signaling pathway. Particularly, in the Hh signal-sending cells, the *mir-310s*...
represses expression of factors regulating Hh ligand production: DHR96, which senses systemic cholesterol levels and promotes Hh release; and Rab23 which, as we propose here, functions in vesicles required for Hh trafficking. In the Hh signal-receiving cells, ttk mRNA encoding the negative Hh signaling transducer and transcription factor, is targeted by the mir-310s. Possibly, targeting of several components of the same signaling pathway is a critical principle of miRNA regulation of stress signaling pathways that should be specifically considered in our understanding of the roles of miRNAs in physiologic and pathophysiologic stress.

Discussion

Here we propose a model for a prompt dietary stress response in which nutritional signals are transduced via miRNAs that act upstream of vital cellular signaling pathways to fine tune their activity and efficiently adapt organismal metabolism to ensure healthy homeostasis (Figure 7). Here we found that the mir-310s, via targeting of multiple Hh pathway components, ensure rapid and robust adjustment of Hh signaling in response to dietary signals. Normally, the capacity of organisms to adapt quickly to changeable food conditions is crucial for their survival since dietary components and food availability can vary rapidly. It is known that adult miRNA mutants rarely show extreme phenotypes in well-controlled laboratory conditions; however, upon stress, a miRNA deficiency frequently has a profound effect on organism survival and adaptability (Bhattacharyya et al. 2006; Leung and Sharp 2010; Mendell and Olson 2012; Edeleva and Shcherbata 2013). Therefore, our interpretations that miRNAs act only upon stress may not be entirely reasonable; their functions may be broader and more basic to control organismal homeostasis. Unique challenges and opportunities for miRNA studies, and in particular for miRNA research focused on the stress response, are to identify the biologically relevant downstream targets regulated by miRNAs, which will allow not only to better understand the mechanisms of stress responses, but also to provide the understanding of how organisms constantly fine tune gene expression to maintain healthy homeostasis in the ever-changing external and internal environments. Here we propose a new workflow that facilitates the identification of miRNA targets and conditions under which studied miRNAs might function.

During embryonic development, miRNAs often act only as fine tuners of gene expression, differentiation guardians, and canalization factors, as embryonic development is extremely well programmed and protected from environmental stimuli and, therefore, it should just be stabilized to succeed (Siegal and Bergman 2002; Hornstein and Shomron 2006; Yatsenko and Shcherbata 2014). However, during adulthood, miRNAs often greatly influence the responses of adult tissues to stressful conditions or hormonal fluctuations (Leung and Sharp 2010; Fagegaltier et al. 2014). To have a profound effect on gene expression, several mechanisms assuring the effectiveness of miRNA-based gene expression regulation have been developed, such as high expression of the miRNA, positive feedback loops, or targeting of multiple components of the critical pathway. For the newly emerged mir-310s family, misexpression would be damaging since the mir-310s have hundreds of putative and several already confirmed critical targets, such as Khc-73, armadillo (arm), and Dystroglycan, deregulation of which could be fatal (Tsurudome et al. 2010; Pancratov et al. 2013; Yatsenko et al. 2014). Positive feedback signaling is also somewhat unlikely because then miRNAs would be expressed in all Hh signal-receiving cells, which, as we have shown, is not the case for the mir-310s. Instead, the mir-310s are expressed dynamically only in some of the Hh signal-sending and signal-receiving cells. Previously it was shown that the mir-310s gene expression is sensitive to nitric oxide levels (Yatsenko et al. 2014), which via nitrosylation of histone deacetylases regulates the cellular epigenetic profile. Epigenetic modifications that play a key role in the regulation of gene expression can also be influenced by both the quality and quantity of the diet (Daniel and Tollefsbol 2015). Based on the previous data, it is logical to hypothesize that the dynamic mir-310s expression in ovaries could also be dependent on specific histone modifications. Currently, it is unknown which signaling induces mir-310s expression in response to deficit of nutrients; however, mir-310s ability to target both the factors required for Hh ligand release in the signal-sending cells (Rab23 and DHR96) and the Hh signal transducer (the transcription factor Ttk) in the signal-receiving cells, ensures that the diet-dependent Hh pathway is securely downregulated upon restrictive diet. While previous data propose that modulation of Hh signaling is a primary dietary stress-response mechanism controlling stem cell proliferation (Horner et al. 2009; Hartman et al. 2013), we show that the mir-310s act upstream of this signaling, demonstrating that miRNAs fine tune a major cell signaling pathway to adjust its strength in the stem cell niche to changing dietary conditions. Even though the miRNAs are generally not well conserved between Drosophila and humans, the processes they regulate are. Therefore, it would be interesting to study whether Hh signaling is also regulated via miRNAs in vertebrates upon diet.

Hh is one of the canonical developmental pathways crucial for the development of a variety of tissues in all bilaterians; thus, finding new components of this pathway is of great importance. We identified the mir-310s as a novel upstream regulatory element of this pathway in Drosophila. Namely, the post-transcriptional control of the expression levels of at least three genes from the Hh pathway (Rab23, DHR96, and ttk) depends on these miRNAs to sustain tissue homeostasis, which has to assume new equilibrium under changing environmental/nutritional conditions. Interestingly, the highly evolutionarily conserved Hh signaling pathway has been shown to play a role in obesity-like fat accumulation in Drosophila and mouse adult stem cells (Pospisilík et al. 2010).

Importantly, we identified a new regulator of Hh signaling in Drosophila, Rab23. Rab proteins are a family of small
GTPases that play key roles in vesicle cargo transport, docking, and fusion and are important for fine tuning of various canonical pathways, safeguarding proper development, tissue morphogenesis, and homeostasis (Zhang et al. 2007). It is known that Rab proteins can have redundant functions (Chan et al. 2011); therefore, deficiency or downregulation of only one of them might not have a dramatic effect on animal viability. Indeed, Rab23 loss of function did not result in any of the analyzed ovarian phenotypes, demonstrating that Rab23 is dispensable for Hh signaling function in the ovary, while its upregulation had an important effect on Hh signaling strength. Based on the proposed Rab23 vertebrate homolog function, Drosophila Rab23 was expected to regulate the trafficking of vesicle-associated components in the Hh signal receiving cells (Evans et al. 2003; Pataki et al. 2010). However, our data demonstrate Rab23-based regulation in the Hh signal sending cells. We propose a new mechanism in which Rab23 has a cell-autonomous role in Hh signal-sending cells in the ovary and that diet-sensitive mir-310s are potent regulators of Rab23 and its downstream trafficking events. Interestingly, Drosophila and human Rab23 are highly evolutionarily conserved (with 59% protein sequence homology) and human Rab23 is a putative target for the human mir-310s orthologs mir-25, mir-32, mir-92a/b/c, mir-363, and mir-367 (Enright et al. 2003; Kheradpour et al. 2007; Betel et al. 2008). miRNA-based control of conserved pathways is also generally conserved between species, implying that their regulatory role could have ancient origins. Therefore it will be important to test whether human Rab23 is regulated via miRNAs as well.

In addition, Rab23 (Wang et al. 2012) and the COPI complex (Beller et al. 2008) have been shown to play a role in lipid homeostasis by affecting lipid droplet size, and the COPI complex also takes part in cholesterol-modified Hh ligand release (Lum et al. 2003; Nybakken et al. 2005; Aikin et al. 2012). Together, our data show that miRNAs can fine-tune cell signaling to tailor adult oogenesis to changing dietary conditions. Since miRNAs usually are capable of targeting regulation of GSC progeny differentiation via targeting of arm levels (Figure 7). This mir-310s-mediated soma–germline communication mechanism (the mir-310s-regulating arm) could additionally be used to coordinate the speed of oogenesis with the nutritional status of the whole organism. Theoretically, the simultaneous management of different signaling pathways via the same miRNAs may aid in coordinating the stress response. In particular, modification of vital cell signaling via miRNAs in response to dietary changes might be commonly implicated in the process of adapting egg production to dietary conditions to ensure sufficient progeny survival.

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Author contributions: I.OÇ. and H.R.S. contributed research design, data acquisition, analysis and interpretation, manuscript draft, and figure design; M.B. and S.E., generation of Rab23::YFP::4xmyc line; and S.K. and H.U., proteomic analysis by mass spectrometry. The authors declare no competing financial interests.

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Hedgehog Signaling Strength Is Orchestrated by the mir-310 Cluster of MicroRNAs in Response to Diet

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Supplemental Information

**Hedgehog signaling strength is orchestrated by the mir-310 cluster of microRNAs in response to diet**

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**Running title:** upon diet, miRNAs modulate Hh signaling

**Keywords:** Drosophila; oogenesis; follicle stem cell; Hedgehog signaling; miRNA; the mir-310s; Rab23; dietary restriction; metabolic stress; Hh ligand
Supplemental Figures

Figure S1

A, A′: Crop size, mm

A′′: µg TAG equivalents /mg protein

B′: Lipid droplets nucleus plasma membrane

C: Days 0-8

D: Eggs laid/fly/day

E: mir-310s mutant egg laying profile in response to starvation

- Control
- mir-310s

Control
- Well-fed
- Starved

mir-310s
- Well-fed
- Starved

0 1 2 3 4 5 6 7 8 days

0 4 8 12 16 20 eggs/laid/fly/day

0.0 0.2 0.4 0.6 0.8 1.0 1.2 normalized to control in well-fed condition
Figure S1. The mir-310s mutant female ovaries respond to protein starvation abnormally

(A, A`) Bright field images of control (w^{1118}) and mir-310s mutant (KT40/KT40) crops dissected from comparably sized females kept under normal conditions. Note the enlarged crop size of mir-310s mutant females (A``) (Table S3).

(B) mir-310s mutant females have abnormal energy metabolism as measured by the total body fat. However, upon nutritional restriction for 10 days, mir-310s mutants accumulate ~2.5-fold more lipids and larger lipid droplets than controls (B`) (Table S3).

(C) In response to nutritional restriction, control females cease egg production after day 4. mir-310s mutant ovaries contain substantial amounts of late egg chambers even after 7-8 days of nutritional restriction (Table S3). mir-310s loss-of-function mutants, similarly to hh (tub-Gal80^{+/+}; bab1-Gal4/UAS-hh at 29°C) and Rab23 (bab1-Gal4/UAS-Rab23) overexpression (data not shown), demonstrate a delayed cessation of egg chamber production after stage 6 in response to starvation.

(D) Note that even under well-fed condition, mir-310s mutant females lay significantly fewer eggs than controls (Table S3).

(E) Egg laying profiles for control and mir-310s mutant females (Table S3).

In (A``), (B), (D), and (E) the data points indicate AVE±SEM (Table S3). Significances were calculated using two-tailed Student’s t-test. *p<0.05, **p<0.005, ***p<0.0005. Scale bar represents 250µm in (A, A`) and 20µm in B`. 
### Supplemental Tables

**Table S1**, related to Figure 1. Proteins significantly deregulated in mir-310s mutants

| CG number | Gene name |
|-----------|-----------|
| **Energy metabolism** | |
| CG10924   | CG10924  |
| CG11594   | CG11594  |
| CG17530   | GstE6    |
| CG2827    | Tal      |
| **CG30360 Mal-A6** | |
| CG31692   | fbp      |
| CG33138   | CG33138  |
| CG3763    | Fbp2     |
| CG4178    | Lsp1beta |
| CG5177    | CG5177   |
| CG6806    | Lsp2     |
| CG8036    | CG8036   |
| CG8094    | Hex-C    |
| **CG8696 LvpH** | |
| CG9092    | Gal      |
| CG9232    | Galt     |
| **Lipid metabolism** | |
| CG10622   | Sucb     |
| CG10932   | CG10932  |
| CG11064   | Rfabg    |
| CG11129   | Yp3      |
| CG11198   | ACC      |
| CG15828   | Apoltp   |
| CG1648    | CG1648   |
| CG1742    | Mgstl    |
| **CG18212 alt** | |
| CG2979    | Yp2      |
| CG2985    | Yp1      |
| **CG3050 Cyp6d5** | |
| CG31150   | crossveinless d |
| CG3481    | Adh      |
| CG3523    | CG3523   |
| CG3524    | v(2)k05816 |
| CG3699    | EG:BACR7A4.14 |
| CG3752    | Aldh     |
| CG4581    | Thiolase |
| CG4729    | CG4729   |
| **CG5170 Dp1** | |
| CG5590    | CG5590   |
| CG5885    | CG5885   |
| CG5958    | CG5958   |
| **CG7400 Fatp** | |
| CG8256    | Gpo-1    |
| CG8628    | CG8628   |
| CG8778    | CG8778   |
| CG9035    | Tapdelta |
| **CG9412 rin** | |
| CG9914    | CG9914   |
| **Protein homeostasis** | |
| CG10236   | LanA     |
| CG10302   | bsf      |
| CG10686   | tral     |
| CG11512   | GstD4    |
| CG11899   | CG11899  |
| CG12163   | CG12163  |
| CG13393   | lethal (2) k12914 |
| CG14715   | CG14715  |
| CG15261   | UK114    |
| CG15369   | CG15369  |
| CG2852    | CG2852   |
| CG3011    | CG3011   |
| CG31198   | CG31198  |
| CG31343   | CG5839   |
| CG33103   | Ppn      |
| CG3926    | Spat     |
| CG3949    | hoip     |
| CG3999    | CG3999   |
| CG4067    | pug      |
| CG4181    | GstD2    |
| CG4463    | Hsp23    |
| CG4659    | Srp54k   |
| CG4916    | me31B    |
| CG4954    | eIF3-S8  |
| CG5064    | Srp68    |
| CG5330    | Nap1     |
| CG5394    | Aats-glupro |
| **Mitochondria** | |
| CG3902    | CG3902   |
| CG10340   | CG10340  |
| CG12203   | CG12203  |
| CG12079   | CG12079  |
| CG12151   | Pdp      |
| CG14757   | CG14757  |
| CG16944   | sesB     |
| CG2286    | ND75     |
| CG32531   | mRpS14   |
| CG3283    | SdhB     |
| CG34073   | mt:ATPase6 |
| CG3566    | CG3566   |
| CG4169    | CG4169   |
| CG4769    | CG4769   |
| **CG5670 Atpalpa** | |
| CG5889    | Men-b    |
| CG6022    | Cchl     |
| CG6455    | CG6455   |
| CG6612    | Adk3     |
| **CG6647 porin** | |
| Gene | Description |
|------|-------------|
| CG6666 | SdhC |
| CG6782 | sea |
| CG6878 | CG6878 |
| CG7580 | CG7580 |
| CG7610 | ATPsyn-gamma |
| CG8479 | opa1-like |
| CG8790 | Dic1 |
| CG8844 | Pdsw |
| CG9090 | CG9090 |
| Nucleotide synthesis | |
| CG11089 | CG11089 |
| CG16758 | CG16758 |
| CG18572 | r |
| CG2194 | su(r) |
| CG31628 | ade3 |
| CG3989 | ade5 |
| CG4584 | dUTPase |
| CG7917 | Nlp |
| CG8132 | CG8132 |
| CG9127 | ade2 |
| CG9193 | mus209 |
| CG9242 | bur |
| CG9674 | CG9674 |
| Muscle | |
| CG10067 | Act57B |
| CG1106 | Gel |
| CG11949 | cora |
| CG12408 | TpnC4 |
| CG15792 | zip |
| CG17927 | Mhc |
| CG17927 | MHC isoforms |
| CG18290 | Act87E |
| CG2184 | Mlc2 |
| CG2981 | TpnC41C |
| CG4183 | Hsp26 |
| CG4466 | Hsp27 |
| CG4843 | Tm2 |
| GC4898 | Tm1 |
| CG5125 | ninaC |
| CG5178 | Act88F |
| CG5596 | Mlc1 |
| CG7107 | up |
| CG7178 | wupA |
| CG7445 | fln |
| CG7478 | Act79B |
| CG7930 | TpnC73F |
| CG9138 | uif |
| CG9432 | l(2)01289 |
| CG9480 | Glycogenin |
| Neural | |
| CG11797 | Obp56a |
| CG12202 | Nat1 |
| CG12908 | Ndg |
| CG15457 | Obp19c |
| CG1618 | cont |
| CG1634 | Nrg |
| CG17029 | CG17029 |
| CG1744 | chp |
| CG17870 | 14-3-3zeta |
| CG18102 | shi |
| CG18111 | Obp99a |
| CG1873 | Ef1alpha100E |
| CG1977 | alpha-Spec |
| CG2028 | Ckalpha |
| CG2297 | Obp44a |
| CG30021 | metro |
| CG32234 | axo |
| CG33950 | trol |
| CG3620 | norpA |
| CG3725 | Ca-P60A, CG3725 |
| CG3747 | Eaat1 |
| CG43079 | nrm |
| CG4609 | fax |
| CG5119 | pAbp |
| CG5711 | Arr1 |
| CG5779 | proPO-A1 |
| CG5779 | proPo |
| CG5870 | beta-Spec |
| CG7088 | bnb |
| CG7576 | Rab3 |
| CG7592 | Obp99b |
| CG8462 | Obp56c |
| CG8663 | nr3v3 |
| CG9206 | Gl |
| CG9261 | Nrv2 |
| Cuticle | |
| CG10112 | Cpr51A |
| CG10287 | Gasp |
| CG12045 | Cpr100A |
| CG17052 | obst-A |
| Histone | |
| CG10638 | CG10638 |
| CG11765 | Prx2540-2 |
| CG12171 | CG12171 |
| CG12405 | Prx2540-1 |
| CG12896 | CG12896 |
| CG18547 | CG18547 |
| CG1982 | Sodh-1 |
| CG3609 | CG3609 |
| CG3835 | EG:87B13 |
| CG6084 | CG6084 |
| CG6776 | GStO3 |
| CG6776 | CG6776 |
| CG7322 | CG7322 |
| CG8503 | CG8503 |
| CG9119 | CG9119 |
| CG9331 | CG9331 |
| His2B | His2B |
| His4 | His4 |
| No association | |
| CG12008 | kst |
| CG10031 | CG10031 |
| CG10527 | CG10527 |
| CG10691 | l(2)37Cc |
| CG10978 | jagn |
| CG11785 | bai |
| CG11920 | CG11920 |
| CG11999 | CG11999 |
| CG12403 | Vha68-1 |
| CG14168 | Zasp67 |
| CG1444 | CG1444 |
| CG1462 | Aph-4 |
| CG14661 | CG14661 |
| CG15081 | l(2)03709 |
| CG15881 | CG15881 |
| CG16884 | BG:DS0180.3 |
| CG16985 | CG16985 |
| CG18591 | SmE |
| CG1885  | CG1885  | CG34026  | CG34026  | CG6851  | Mtxh   |
|-------|--------|----------|----------|--------|--------|
| CG2082 | CG2082 | CG34215  | CG34215  | CG6917  | Est-6  |
| CG2216 | FerlHCH| CG42314  | PMCA     | CG6950  | CG6950 |
| CG2233 | CG2233 | CG4239   | CG4239   | CG7646  | CG7646 |
| CG2310 | CG2310 | CG5945   | CG5945   | CG8108  | CG8108 |
| CG2943 | CG2943 | CG6214   | MRP      | CG8790  | CG8790 |
| CG30222| CG30222| CG6544   | fau      | CG9297  | CG9297 |
| CG3082 | l(2)k09913| CG6702 | Cbp53E   |         |        |
| CG31195| CG31195| CG6815   | bor      |         |        |

**Putative mir-310s target**
Table S2, related to Figure 1. Relative mRNA expression levels of the starvation-sensitive genes upon mir-310s deficit and/or nutritional stress

| Genotype/Condition | Target Gene | Cₜ AVE±SEMᵇ | ΔCₜ AVE±SEMᵇ | ΔΔCₜ AVE±SEMᵇ | Relative mRNA levelᵇᶜ AVE±SEMᵇ | log₁₀ Relative mRNA level AVE±SEMᵇ |
|--------------------|-------------|-------------|---------------|----------------|----------------------------------|----------------------------------|
| **Plate 1**        |             |             |               |                |                                  |                                  |
| Control (w¹¹¹⁸)    | Act88F      | 2.76E+01    | ±5.57E-02     | 9.47           | 3.18E-07           | ±5.57E-02                 | 1.00                                 | -9.57E-08                             | ±6.27E-02                               | ±1.68E-02                           |
| well-fed           |             | 2.16E+01    | ±1.79E-02     | 3.58           | -5.88              | ±1.79E-02                 | 5.90E+01                             | ±7.30E-01                             | ±3.80E-02                               | ±1.77                                 |
| starved            |             | 2.50E+01    | ±3.11E-02     | 6.45           | -3.02              | ±3.11E-02                 | 8.09                                 | ±1.73E-01                             | ±3.16E-02                               | ±5.38E-03                           |
| mir-310s (KT40/KT40) | well-fed   | 2.40E+01    | ±9.20E-03     | 5.79           | -3.68              | ±9.20E-03                 | 1.28E+01                             | ±8.18E-02                             | ±1.79E-03                               | ±1.11                                 |
| starved            |             | 2.40E+01    | ±9.20E-03     | 5.79           | -3.68              | ±9.20E-03                 | 1.28E+01                             | ±8.18E-02                             | ±1.79E-03                               | ±1.11                                 |
| Control (w¹¹¹⁸)    | ade2        | 2.32E+01    | ±3.16E-02     | 4.70           | 1.59E-07           | ±3.16E-02                 | 1.00                                 | ±2.18E-02                             | ±4.78E-08                               | ±9.52E-03                           |
| well-fed           |             | 2.32E+01    | ±4.70E-02     | 5.17           | 4.65E-01           | ±4.70E-02                 | 7.25E-01                             | ±2.35E-02                             | ±1.40E-01                               | ±1.42E-02                           |
| starved            |             | 2.19E+01    | ±3.21E-02     | 3.32           | -1.38              | ±2.31E-02                 | 2.61                                 | ±4.15E-02                             | ±6.95E-03                               | ±4.30E-06                           |
| mir-310s (KT40/KT40) | well-fed   | 2.25E+01    | ±1.49E-02     | 4.36           | -3.45E-01          | ±1.49E-02                 | 1.27                                 | ±1.32E-02                             | ±4.49E-03                               | ±4.49E-03                           |
| starved            |             | 2.25E+01    | ±1.49E-02     | 4.36           | -3.45E-01          | ±1.49E-02                 | 1.27                                 | ±1.32E-02                             | ±4.49E-03                               | ±4.49E-03                           |
| Control (w¹¹¹⁸)    | ade3        | 2.34E+01    | ±1.77E-02     | 5.21           | -1.59E-07          | ±1.77E-02                 | 1.00                                 | ±1.22E-02                             | ±4.78E-08                               | ±5.33E-03                           |
| well-fed           |             | 2.49E+01    | ±2.39E-02     | 6.91           | 1.70               | ±2.39E-02                 | 3.09E-01                             | ±5.09E-03                             | ±5.11E-01                               | ±7.19E-03                           |
| starved            |             | 2.27E+01    | ±1.78E-02     | 4.15           | -1.06              | ±1.78E-02                 | 2.08                                 | ±2.56E-02                             | ±3.18E-01                               | ±5.36E-03                           |
| mir-310s (KT40/KT40) | well-fed   | 2.39E+01    | ±2.81E-02     | 5.73           | 5.16E-01           | ±2.81E-02                 | 6.99E-01                             | ±1.37E-02                             | ±1.55E-01                               | ±8.46E-03                           |
| starved            |             | 2.39E+01    | ±2.81E-02     | 5.73           | 5.16E-01           | ±2.81E-02                 | 6.99E-01                             | ±1.37E-02                             | ±1.55E-01                               | ±8.46E-03                           |
| Control (w¹¹¹⁸)    | Arr1        | 2.30E+01    | ±9.35E-03     | 4.80           | 0.00               | ±9.35E-03                 | 1.00                                 | ±6.49E-03                             | 0.00                                  | ±2.82E-03                           |
| well-fed           |             | 2.03E+01    | ±4.21E-02     | 2.27           | -1.83              | ±7.03E-01                 | 3.56                                 | ±1.49                                 | ±5.52E-01                               | ±1.27E-02                           |
| starved            |             | 2.28E+01    | ±1.09E-01     | 4.27           | -9.41E-01          | ±5.17E-01                 | 1.92                                 | ±8.63E-01                             | ±2.35E-01                               | 2.83E-01                             |
| mir-310s (KT40/KT40) | well-fed   | 2.14E+01    | ±3.34E-02     | 3.19           | -8.18E-01          | ±8.12E-01                 | 1.76                                 | ±8.43E-01                             | ±2.44E-01                               | -4.71E-01                            |
| starved            |             | 2.14E+01    | ±3.34E-02     | 3.19           | -8.18E-01          | ±8.12E-01                 | 1.76                                 | ±8.43E-01                             | ±2.44E-01                               | -4.71E-01                            |
| Control (w¹¹¹⁸)    | CG3699      | 2.39E+01    | ±5.05E-02     | 5.72           | -1.59E-07          | ±5.05E-02                 | 1.00                                 | ±3.55E-02                             | ±4.78E-08                               | ±1.52E-02                           |
| well-fed           |             | 2.53E+01    | ±3.47E-02     | 7.28           | 1.56               | ±3.47E-02                 | 3.38E-03                             | ±6.55E-03                             | ±4.71E-01                               | ±8.49E-03                           |
| starved            |             | 2.53E+01    | ±3.47E-02     | 7.28           | 1.56               | ±3.47E-02                 | 3.38E-03                             | ±6.55E-03                             | ±4.71E-01                               | ±8.49E-03                           |
| mir-310s (KT40/KT40) well-fed | 2.50E+01 ±7.56E-03 | 6.43 ±3.05E-02 | 7.14E-01 ±7.56E-03 | 6.09E-01 ±3.19E-03 | -2.15E-01 ±2.28E-03 |
| mir-310s (KT40/KT40) starved | 2.52E+01 ±7.45E-03 | 7.03 ±1.06E-02 | 1.31 ±7.45E-03 | 4.03E-01 ±2.08E-03 | -3.95E-01 ±2.24E-03 |
| Control (w1118) well-fed | 2.29E+01 ±1.99E-03 | 4.78 ±2.89E-02 | 1.59E-07 ±1.99E-03 | 1.00 ±1.38E-03 | -4.78E-08 ±5.99E-04 |
| Control (w1118) starved | 2.43E+01 ±2.56E-02 | 6.32 ±3.26E-02 | 1.54 ±2.56E-02 | 3.44E-01 ±6.17E-03 | -4.63E-01 ±7.72E-03 |
| mir-310s (KT40/KT40) well-fed | 2.26E+01 ±1.72E-02 | 4.04 ±3.41E-02 | -7.34E-01 ±1.72E-02 | 1.66 ±1.98E-02 | 2.21E-01 ±5.16E-03 |
| mir-310s (KT40/KT40) starved | 2.33E+01 ±1.23E-02 | 5.18 ±1.45E-02 | 3.97E-01 ±1.23E-02 | 7.60E-01 ±6.46E-03 | -1.19E-01 ±3.71E-03 |
| Control (w1118) well-fed | 1.82E+01 ±2.88E-02 | 0.00 ±2.88E-02 |  |  |  |
| Control (w1118) starved | 1.80E+01 ±2.02E-02 | 6.36E-07 ±2.02E-02 |  |  |  |
| mir-310s (KT40/KT40) well-fed | 1.86E+01 ±2.95E-02 | 1.91E-06 ±2.95E-02 |  |  |  |
| mir-310s (KT40/KT40) starved | 1.82E+01 ±7.58E-03 | 6.36E-07 ±7.58E-03 |  |  |  |
| CG3902 |  |  |  |  |  |
| CG3999 |  |  |  |  |  |
| Rpl32 | 1.82E+01 ±2.88E-02 | 0.00 ±2.88E-02 |  |  |  |
| No Reverse Transcriptase | 3.34E+01 ±2.44E-01 | 1.53E+01 ±2.44E-01 | 1.53E+01 ±2.44E-01 | 2.33E-05 ±4.68E-06 | -4.60 ±7.35E-02 |
| Control (w1118) starved | 3.30E+01 ±2.87E-01 | 1.50E+01 ±2.87E-01 | 1.50E+01 ±2.87E-01 | 3.06E-05 ±5.89E-06 | -4.51 ±8.65E-02 |
| mir-310s (KT40/KT40) well-fed | 3.28E+01 ±1.09E-01 | 1.43E+01 ±1.09E-01 | 1.43E+01 ±1.09E-01 | 5.06E-05 ±3.98E-06 | -4.30 ±3.29E-02 |
| mir-310s (KT40/KT40) starved | 3.37E+01 ±1.36E-01 | 1.56E+01 ±1.36E-01 | 1.56E+01 ±1.36E-01 | 2.08E-05 ±1.95E-06 | -4.68 ±4.10E-02 |
| Plate 2 | 2.69E+01 ±1.07E-02 | 9.08 ±2.33E-02 | -6.36E-07 ±1.07E-02 | 1.00 ±7.44E-03 | 1.91E-07 ±3.22E-03 |
| Control (w1118) well-fed | 2.85E+01 ±2.78E-02 | 1.08E+01 ±2.98E-02 | 1.72 ±2.78E-02 | 3.04E-01 ±5.90E-03 | -5.17E-01 ±8.36E-03 |
| Control (w1118) starved | 2.68E+01 ±3.90E-02 | 8.55 ±4.82E-02 | -5.30E-01 ±3.90E-02 | 1.44 ±3.95E-02 | 1.60E-01 ±1.17E-02 |
| mir-310s (KT40/KT40) well-fed | 2.76E+01 ±4.55E-02 | 9.69 ±4.81E-02 | 6.09E-01 ±4.55E-02 | 6.56E-01 ±2.10E-02 | -1.83E-01 ±1.37E-02 |
| mir-310s (KT40/KT40) starved | 3.08E+01 ±4.14E-02 | 1.30E+01 ±4.63E-02 | -9.54E-07 ±4.14E-02 | 1.00 ±2.83E-02 | 2.87E-07 ±1.25E-02 |
| Control (w1118) well-fed | 3.19E+01 ±2.46E-02 | 1.42E+01 ±2.69E-02 | 1.20 ±2.46E-02 | 4.34E-01 ±7.41E-03 | -3.62E-01 ±7.42E-03 |
| mir-310s (KT40/KT40) | 3.08E+01 ± 2.57E-02 | 1.25E+01 ± 3.83E-02 | -4.46E-01 ± 2.57E-02 | 1.36 ± 2.44E-02 | 1.34E-01 ± 7.43E-03 |
| mir-310s (KT40/KT40) | 3.06E+01 ± 5.07E-02 | 1.27E+01 ± 5.30E-02 | -2.91E-01 ± 5.07E-02 | 1.22 ± 4.31E-02 | 8.76E-02 ± 1.53E-02 |
| Control (w1118) | 2.22E+01 ± 2.57E-02 | 4.41 ± 3.30E-02 | -7.95E-07 ± 2.57E-02 | 1.00 ± 1.80E-02 | 2.39E-07 ± 7.33E-03 |
| Control (w1118) | 2.30E+01 ± 1.08E-02 | 5.33 ± 1.54E-02 | 9.29E-01 ± 1.08E-02 | 5.25E-01 ± 3.96E-03 | -2.80E-01 ± 3.27E-03 |
| Control (w1118) | 2.15E+01 ± 9.71E-03 | 3.30 ± 3.00E-02 | -1.10 ± 9.71E-03 | 2.15 ± 1.44E-02 | 3.32E-01 ± 2.92E-03 |
| mir-310s (KT40/KT40) | 2.30E+01 ± 1.41E-02 | 5.10 ± 2.10E-02 | 6.99E-01 ± 1.41E-02 | 6.16E-01 ± 6.07E-03 | -2.10E-01 ± 4.26E-03 |
| Control (w1118) | 3.29E+01 ± 8.65E-03 | 1.51E+01 ± 2.24E-02 | -3.18E-07 ± 8.65E-03 | 1.00 ± 5.97E-03 | 9.57E-08 ± 2.60E-03 |
| Control (w1118) | 3.17E+01 ± 1.71E-01 | 1.40E+01 ± 1.72E-01 | -1.12 ± 1.71E-01 | 2.18 ± 2.56E-01 | 3.38E-01 ± 5.15E-02 |
| mir-310s (KT40/KT40) | 3.29E+01 ± 8.31E-02 | 1.47E+01 ± 8.78E-02 | -4.16E-01 ± 8.31E-02 | 1.33 ± 7.67E-02 | 1.25E-01 ± 2.50E-02 |
| mir-310s (KT40/KT40) | 3.11E+01 ± 8.40E-02 | 1.32E+01 ± 8.54E-02 | -1.86 ± 8.40E-02 | 3.64 ± 2.06E-01 | 5.61E-01 ± 2.53E-02 |
| Control (w1118) | 3.60E+01 ± 1.55E-01 | 1.82E+01 ± 1.57E-01 | 0.00 ± 1.55E-01 | 1.00 ± 1.03E-01 | 0.00 ± 4.68E-02 |
| Control (w1118) | 3.48E+01 ± 6.19E-02 | 1.70E+01 ± 6.29E-02 | -1.13 ± 6.19E-02 | 2.20 ± 9.23E-02 | 3.41E-01 ± 1.86E-02 |
| mir-310s (KT40/KT40) | 3.63E+01 ± 5.23E-02 | 1.81E+01 ± 5.95E-02 | -8.92E-02 ± 5.23E-02 | 1.06 ± 3.80E-02 | 2.69E-02 ± 1.57E-02 |
| mir-310s (KT40/KT40) | 3.49E+01 ± 1.49E-01 | 1.71E+01 ± 1.50E-01 | -1.13 ± 1.49E-01 | 2.18 ± 2.27E-01 | 3.39E-01 ± 4.48E-02 |
| Control (w1118) | 2.19E+01 ± 1.06E-02 | 4.11 ± 2.32E-02 | -7.95E-07 ± 1.06E-02 | 1.00 ± 7.30E-03 | 2.39E-07 ± 3.18E-03 |
| Control (w1118) | 2.30E+01 ± 1.63E-02 | 5.32 ± 1.96E-02 | 1.22 ± 1.63E-02 | 4.30E-01 ± 4.86E-03 | -3.67E-01 ± 4.91E-03 |
| mir-310s (KT40/KT40) | 2.27E+01 ± 1.47E-02 | 4.50 ± 3.19E-02 | 3.92E-01 ± 1.47E-02 | 7.62E-01 ± 7.78E-03 | -1.18E-01 ± 4.41E-03 |
| mir-310s (KT40/KT40) | 2.34E+01 ± 2.02E-02 | 5.52 ± 2.55E-02 | 1.41 ± 2.02E-02 | 3.76E-01 ± 5.23E-03 | -4.24E-01 ± 6.08E-03 |
| Control (w1118) | 1.78E+01 ± 2.07E-02 | -6.36E-07 ± 2.07E-02 | | | |
| Control (w1118) | 1.77E+01 ± 1.09E-02 | 0.00 ± 1.09E-02 | | | |
| mir-310s (KT40/KT40) | 1.82E+01 ± 2.84E-02 | -1.27E-06 ± 2.84E-02 | | | |
| mir-310s (KT40/KT40) | 1.79E+01 ±1.55E-02 | 6.36E-07 ±1.55E-02 |
|-----------------------|---------------------|---------------------|
| Control (w1118) well-fed | 3.28E+01 ±1.19E-01 | 1.50E+01 ±1.19E-01 |
| Control (w1118) starved | 3.28E+01 ±1.03E-01 | 1.51E+01 ±1.03E-01 |
| mir-310s (KT40/KT40) well-fed | 3.22E+01 ±9.10E-02 | 1.40E+01 ±9.10E-02 |
| mir-310s (KT40/KT40) starved | 3.27E+01 ±2.33E-01 | 1.49E+01 ±2.33E-01 |

No Reverse Transcriptase

| Control (w1118) well-fed | 2.54E+01 ±1.38E-02 | 7.51 ±2.92E-02 |
| Control (w1118) starved | 2.36E+01 ±1.64E-03 | 5.80 ±9.31E-03 |
| mir-310s (KT40/KT40) well-fed | 2.59E+01 ±3.60E-02 | 7.76 ±1.04E-01 |
| mir-310s (KT40/KT40) starved | 2.43E+01 ±1.91E-02 | 6.43 ±2.24E-02 |

Plate 3

| Control (w1118) well-fed | 3.45E+01 ±7.64E-02 | 1.66E+01 ±8.06E-02 |
| Control (w1118) starved | 3.15E+01 ±3.45E-02 | 1.37E+01 ±3.57E-02 |
| mir-310s (KT40/KT40) well-fed | 3.20E+01 ±5.19E-02 | 1.38E+01 ±1.11E-01 |
| mir-310s (KT40/KT40) starved | 3.01E+01 ±1.61E-02 | 1.23E+01 ±2.00E-02 |

| Control (w1118) well-fed | 3.25E+01 ±8.92E-02 | 1.46E+01 ±9.28E-02 |
| Control (w1118) starved | 3.29E+01 ±1.73E-02 | 1.51E+01 ±1.96E-02 |
| mir-310s (KT40/KT40) well-fed | 2.80E+01 ±9.14E-03 | 9.84 ±9.80E-02 |
| mir-310s (KT40/KT40) starved | 2.62E+01 ±3.01E-02 | 8.39 ±3.24E-02 |

| Control (w1118) well-fed | 3.18E+01 ±1.13E-01 | 1.39E+01 ±1.16E-01 |
| Control (w1118) starved | 2.79E+01 ±1.96E-02 | 1.01E+01 ±2.17E-02 |
| mir-310s (KT40/KT40) well-fed | 2.78E+01 ±4.13E-02 | 9.63 ±1.06E-01 |
| Sample                          | 2.84E+01 ±3.03E-02 | 1.06E±01 ±3.25E-02 | -3.31 ±3.03E-02 | 9.93 ±2.10E-01 p<0.01 Control well-fed | 9.97E-01 ±9.11E-03 |
|--------------------------------|---------------------|--------------------|----------------|--------------------------------------|-------------------|
| mir-310s (KT40/KT40) starved  | 2.76E+01 ±6.26E-02 | 9.77 ±6.77E-02    | -3.18E-07 ±6.26E-02 | 1.00 ±4.26E-02 p<0.01 Control well-fed | 9.57E-08 ±1.89E-02 |
| Control (w1118) starved       | 2.79E+01 ±4.60E-02 | 1.01E+01 ±4.69E-02 | 3.15E-01 ±4.60E-02 | 8.04E-01 ±2.55E-02 p<0.01 Control well-fed | -9.50E-02 ±1.38E-02 |
| mir-310s (KT40/KT40) starved  | 2.86E+01 ±1.76E-02 | 1.04E+01 ±9.91E-02 | 6.79E-01 ±1.76E-02 | 6.25E-01 ±7.62E-03 p<0.01 Control well-fed | -2.04E-01 ±5.31E-03 |
| Control (w1118) starved       | 2.80E+01 ±1.89E-02 | 1.01E+01 ±2.23E-02 | 3.70E-01 ±1.89E-02 | 7.74E-01 ±1.02E-02 p<0.01 Control well-fed | -1.11E-01 ±5.68E-03 |
| mir-310s (KT40/KT40) starved  | 2.99E+01 ±4.09E-02 | 1.20E+01 ±4.83E-02 | 3.18E-07 ±4.09E-02 | 1.00 ±2.79E-02 p<0.01 Control well-fed | -9.57E-08 ±1.23E-02 |
| Control (w1118) starved       | 2.61E+01 ±1.95E-02 | 8.34 ±2.16E-02    | -3.67 ±1.95E-02 | 1.27E+01 ±1.73E-01 p<0.01 Control well-fed | 1.10 ±5.88E-03    |
| mir-310s (KT40/KT40) starved  | 2.90E+01 ±2.72E-02 | 1.08E+01 ±1.01E-01 | -1.16 ±2.72E-02 | 2.23 ±4.20E-02 p<0.01 Control well-fed | 3.49E-01 ±8.20E-03 |
| Control (w1118) starved       | 2.76E+01 ±1.90E-02 | 9.77 ±2.24E-02    | -2.24 ±1.90E-02 | 4.72 ±6.19E-02 p<0.01 Control well-fed | 6.74E-01 ±5.72E-03 |
| mir-310s (KT40/KT40) starved  | 1.79E+01 ±2.57E-02 | 0.00 ±2.57E-02    | -1.62E ±2.57E-02 | -4.55 ±2.07E-02 p<0.01 Control well-fed | 3.04E-02           |
| Control (w1118) starved       | 1.78E+01 ±9.16E-03 | 6.36E-07 ±9.16E-03 | -1.16E ±9.16E-03 | -4.54 ±3.04E-02 p<0.01 Control well-fed | 3.04E-02           |
| mir-310s (KT40/KT40) starved  | 1.81E+01 ±9.76E-02 | 0.00 ±9.76E-02    | -1.16E ±9.76E-02 | -4.27 ±8.06E-02 p<0.01 Control well-fed | 3.04E-02           |
| Control (w1118) starved       | 1.78E+01 ±1.18E-02 | 0.00 ±1.18E-02    | -1.16E ±1.18E-02 | -4.57 ±2.98E-03 p<0.01 Control well-fed | 3.04E-02           |

No Reverse Transcriptase

| Sample                          | 3.30E+01 ±6.88E-02 | 1.51E+01 ±6.88E-02 | 1.51E+01 ±6.88E-02 | 2.81E-05 ±1.34E-06 | -4.55 ±2.07E-02 |
|--------------------------------|---------------------|--------------------|--------------------|----------------|-----------------|
| Control (w1118) well-fed        | 3.29E+01 ±1.01E-01 | 1.51E+01 ±1.01E-01 | 1.51E+01 ±1.01E-01 | 2.90E-05 ±2.03E-06 | -4.54 ±3.04E-02 |
| Control (w1118) starved         | 3.23E+01 ±2.68E-01 | 1.42E+01 ±2.68E-01 | 1.42E+01 ±2.68E-01 | 5.32E-05 ±9.93E-06 | -4.27 ±8.06E-02 |
| mir-310s (KT40/KT40) well-fed   | 3.30E+01 ±9.89E-03 | 1.52E+01 ±9.89E-03 | 1.52E+01 ±9.89E-03 | 2.67E-05 ±1.83E-07 | -4.57 ±2.98E-03 |
| mir-310s (KT40/KT40) starved    | 2.55E+01 ±3.46E-02 | 8.26 ±3.85E-02    | -3.18E-07 ±3.46E-02 | 1.00 ±2.42E-02 p<0.01 Control well-fed | 9.57E-08 ±1.04E-02 |

Plate 4

| Sample                          | 2.56E+01 ±2.51E-02 | 8.55 ±2.63E-02    | 2.91E-01 ±2.51E-02 | 8.17E-01 ±1.43E-02 p<0.01 Control well-fed | -8.77E-02 ±7.57E-03 |
|--------------------------------|---------------------|--------------------|--------------------|----------------|-----------------|
| Control (w1118) starved         | 2.54E+01 ±1.23E-02 | 7.72 ±3.97E-02    | -5.38E-01 ±1.23E-02 | 1.45 ±1.24E-02 p<0.01 Control well-fed | 1.62E-01 ±3.71E-03 |
| mir-310s (KT40/KT40) | Lsp1beta | Lsp2 | LypH | Mgstl | mus209 |
|-----------------------|----------|------|-----|-------|-------|
| starved               |          |      |     |       |       |
| Control (w1118)       |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.59E+01 ±1.08E-02    | 8.72 ±1.58E-02 | 4.55E-01 ±1.08E-02 | 7.30E-01 ±5.46E-03 | -1.37E-01 ±3.26E-03 |
| Control (w1119)       |          |      |     |       |       |
| starved               |          |      |     |       |       |
| 2.69E+01 ±3.96E-02    | 9.65 ±4.31E-02 | -6.36E-07 ±3.96E-02 | 1.00 ±2.73E-02 | 1.91E-07 ±1.19E-02 |
| mir-310s (KT40/KT40) |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 3.17E+01 ±9.43E-03    | 1.47E+01 ±1.23E-02 | 5.08 ±9.43E-03 | 2.95E-02 ±1.93E-04 | -1.53 ±2.84E-03 |
| Control (w1118)       |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.39E+01 ±2.93E-02    | 6.26 ±4.78E-02 | -3.39 ±2.93E-02 | 1.05E+01 ±2.12E-04 | 1.02 ±8.82E-03 |
| Control (w1119)       |          |      |     |       |       |
| starved               |          |      |     |       |       |
| 2.40E+01 ±2.88E-02    | 6.85 ±3.11E-02 | -2.80 ±2.88E-02 | 6.98 ±1.38E-01 | 8.44E-01 ±8.67E-03 |
| Control (w1118)       |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 1.98E+01 ±1.86E-02    | 2.57 ±2.52E-02 | -3.63E-07 ±1.86E-02 | 1.00 ±1.28E-02 | 1.91E-07 ±5.61E-03 |
| Control (w1119)       |          |      |     |       |       |
| starved               |          |      |     |       |       |
| 2.87E+01 ±4.05E-02    | 1.17E+01 ±4.12E-02 | 9.17 ±4.05E-02 | 1.74E-03 ±4.95E-05 | -2.76 ±1.22E-02 |
| mir-310s (KT40/KT40) |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.19E+01 ±2.81E-03    | 4.30 ±3.78E-02 | 1.72 ±2.81E-03 | 3.03E+01 ±5.89E-04 | -5.19E-01 ±8.45E-04 |
| Control (w1118)       |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.34E+01 ±2.49E-02    | 6.17 ±2.74E-02 | 3.59 ±2.49E-02 | 8.28E+02 ±1.44E-03 | -1.08 ±7.49E-03 |
| Control (w1119)       |          |      |     |       |       |
| starved               |          |      |     |       |       |
| 2.17E+01 ±4.82E-02    | 4.53 ±5.10E-02 | -4.77E-07 ±4.82E-02 | 1.00 ±3.29E-02 | 1.44E-07 ±1.45E-02 |
| Control (w1118)       |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.18E+01 ±2.57E-02    | 4.76 ±2.69E-02 | 2.35E-01 ±2.57E-02 | 8.50E-01 ±1.52E-02 | -7.06E-02 ±7.74E-03 |
| Control (w1119)       |          |      |     |       |       |
| starved               |          |      |     |       |       |
| 2.23E+01 ±2.06E-02    | 4.63 ±4.30E-02 | 1.01E-01 ±2.06E-02 | 9.32E-01 ±1.34E-02 | -3.04E-02 ±6.21E-03 |
| mir-310s (KT40/KT40) |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.29E+01 ±2.60E-03    | 5.74 ±1.19E-02 | 1.21 ±2.60E-03 | 4.32E-01 ±7.80E-04 | -3.64E-01 ±7.84E-04 |
| Control (w1118)       |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.29E+01 ±1.60E-02    | 5.71 ±2.33E-02 | -4.77E-07 ±1.60E-02 | 1.00 ±1.11E-02 | 1.44E-07 ±4.82E-03 |
| Control (w1119)       |          |      |     |       |       |
| starved               |          |      |     |       |       |
| 2.34E+01 ±1.61E-02    | 6.38 ±1.79E-02 | 6.76E-01 ±1.61E-02 | 6.26E-01 ±6.96E-03 | -2.04E-01 ±4.83E-03 |
| mir-310s (KT40/KT40) |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.24E+01 ±5.28E-03    | 4.78 ±3.81E-02 | -9.32E-01 ±5.28E-03 | 1.91 ±6.99E-03 | 2.80E-01 ±1.59E-03 |
| Control (w1118)       |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.24E+01 ±1.72E-01    | 5.22 ±1.72E-01 | -4.89E-01 ±1.72E-01 | 1.40 ±1.76E-01 | 1.47E-01 ±5.17E-02 |
| Control (w1119)       |          |      |     |       |       |
| starved               |          |      |     |       |       |
| 2.19E+01 ±1.36E-02    | 4.70 ±2.17E-02 | -6.36E-07 ±1.36E-02 | 1.00 ±9.37E-03 | 1.91E-07 ±4.08E-03 |
| Control (w1118)       |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.44E+01 ±9.25E-03    | 7.39 ±1.21E-02 | 2.69 ±9.25E-03 | 1.55E-01 ±9.93E-04 | -8.10E-01 ±2.78E-03 |
| Control (w1119)       |          |      |     |       |       |
| starved               |          |      |     |       |       |
| 2.19E+01 ±5.19E-03    | 4.24 ±3.81E-02 | -4.61E-01 ±5.19E-03 | 1.38 ±4.96E-03 | 1.39E-01 ±1.56E-03 |
| mir-310s (KT40/KT40) |          |      |     |       |       |
| well-fed              |          |      |     |       |       |
| 2.34E+01 ±3.90E-03    | 6.20 ±1.22E-02 | 1.51 ±3.90E-03 | 3.52E-01 ±9.51E-04 | -4.54E-01 ±1.18E-03 |
|                     | Control (w¹¹¹⁸) well-fed | Control (w¹¹¹⁸) starved | mir-310s (KT40/KT40) well-fed | mir-310s (KT40/KT40) starved |
|---------------------|--------------------------|-------------------------|------------------------------|-------------------------------|
| **Rpl32**           |                          |                         |                              |                               |
|                     | 1.72E+01 ±1.69E-02       | -6.36E-07 ±1.69E-02     | 1.70E+01 ±7.88E-03           | 6.36E-07 ±7.88E-03            |
|                     | 1.76E+01 ±3.77E-02       | 0.00 ±3.77E-02          | 1.76E+01 ±1.16E-02           | 1.27E-06 ±1.16E-02            |

**No Reverse Transcriptase**

|                     | Control (w¹¹¹⁸) well-fed | Control (w¹¹¹⁸) starved | mir-310s (KT40/KT40) well-fed | mir-310s (KT40/KT40) starved |
|---------------------|--------------------------|-------------------------|------------------------------|-------------------------------|
| **Rpl32**           |                          |                         |                              |                               |
|                     | 3.19E+01 ±1.37E-02       | 1.47E+01 ±3.55E-02      | -3.18E-07 ±3.37E-02          | 1.00 ±2.36E-02                |
|                     | 3.30E+01 ±1.33E+01       | 1.33E+01 ±5.16E-02      | -1.33E-01 ±1.68E-02          | 1.52 ±3.89E-02               |
|                     | 3.03E+01 ±1.28E-02       | 1.27E+01 ±3.78E-02      | -4.28E-01 ±1.28E-02          | 1.35 ±1.19E-02               |

**Plate 5**

|                     | Control (w¹¹¹⁸) well-fed | Control (w¹¹¹⁸) starved | mir-310s (KT40/KT40) well-fed | mir-310s (KT40/KT40) starved |
|---------------------|--------------------------|-------------------------|------------------------------|-------------------------------|
| **Obp44a**          |                          |                         |                              |                               |
|                     | 2.69E+01 ±3.37E-02       | 8.55 ±3.55E-02          | -3.18E-07 ±3.37E-02          | 1.00 ±2.36E-02                |
|                     | 2.67E+01 ±1.68E-02       | 8.41 ±5.16E-02          | -1.33E-01 ±1.68E-02          | 1.52 ±3.89E-02               |
|                     | 2.68E+01 ±3.72E-02       | 7.95 ±5.92E-02          | -6.01E-01 ±3.72E-02          | 1.35 ±1.19E-02               |
|                     | 2.66E+01 ±1.28E-02       | 8.12 ±3.78E-02          | -4.28E-01 ±1.28E-02          |                                |

**Obp56a**

|                     | Control (w¹¹¹⁸) well-fed | Control (w¹¹¹⁸) starved | mir-310s (KT40/KT40) well-fed | mir-310s (KT40/KT40) starved |
|---------------------|--------------------------|-------------------------|------------------------------|-------------------------------|
|                     | 2.57E+01 ±2.06E-02       | 7.32 ±2.34E-02          | -1.59E-07 ±2.06E-02          | 1.00 ±1.43E-02               |
|                     | 2.49E+01 ±7.92E-03       | 6.70 ±4.94E-02          | -6.17E-01 ±7.92E-03          | 1.53 ±8.39E-03               |
|                     | 2.79E+01 ±2.00E-02       | 9.05 ±5.02E-02          | 1.73 ±2.00E-02               | 3.02E-01 ±4.16E-03           |
|                     | 2.75E+01 ±5.34E-02       | 9.00 ±6.41E-02          | 1.68 ±5.34E-02               | 3.11E-01 ±1.17E-02           |

**Obp56e**

|                     | Control (w¹¹¹⁸) well-fed | Control (w¹¹¹⁸) starved | mir-310s (KT40/KT40) well-fed | mir-310s (KT40/KT40) starved |
|---------------------|--------------------------|-------------------------|------------------------------|-------------------------------|
|                     | 2.46E+01 ±3.45E-02       | 6.25 ±3.63E-02          | 0.00 ±3.45E-02               | 1.00 ±2.41E-02               |
|                     | 2.56E+01 ±1.69E-02       | 7.36 ±5.16E-02          | 1.11 ±1.69E-02               | 4.64E-01 ±5.41E-03           |
|                     | 2.76E+01 ±1.19E-02       | 8.76 ±4.76E-02          | 2.51 ±1.19E-02               | 1.76E-01 ±1.45E-03           |
|                     | 2.69E+01 ±2.14E-02       | 8.36 ±4.15E-02          | 2.11 ±2.14E-02               | 2.32E-01 ±3.47E-03           |

**Plate 5**

|                     | Control (w¹¹¹⁸) well-fed | Control (w¹¹¹⁸) starved | mir-310s (KT40/KT40) well-fed | mir-310s (KT40/KT40) starved |
|---------------------|--------------------------|-------------------------|------------------------------|-------------------------------|
|                     | 2.55E±01 ±1.69E-02       | 7.36 ±5.16E-02          | 1.11 ±1.69E-02               | 4.64E-01 ±5.41E-03           |
|                     | 2.76E±01 ±1.19E-02       | 8.76 ±4.76E-02          | 2.51 ±1.19E-02               | 1.76E-01 ±1.45E-03           |
|                     | 2.69E±01 ±2.14E-02       | 8.36 ±4.15E-02          | 2.11 ±2.14E-02               | 2.32E±01 ±3.47E-03           |

|                     | Control (w¹¹¹⁸) well-fed | Control (w¹¹¹⁸) starved | mir-310s (KT40/KT40) well-fed | mir-310s (KT40/KT40) starved |
|---------------------|--------------------------|-------------------------|------------------------------|-------------------------------|
|                     | 2.55E±01 ±1.69E-02       | 7.36 ±5.16E-02          | 1.11 ±1.69E-02               | 4.64E-01 ±5.41E-03           |
|                     | 2.76E±01 ±1.19E-02       | 8.76 ±4.76E-02          | 2.51 ±1.19E-02               | 1.76E-01 ±1.45E-03           |
|                     | 2.69E±01 ±2.14E-02       | 8.36 ±4.15E-02          | 2.11 ±2.14E-02               | 2.32E±01 ±3.47E-03           |
|              | Obp99b               | Obst-A               | pro-PO-AI          | Rpl32               | No Reverse Transcriptase |
|--------------|----------------------|----------------------|--------------------|----------------------|--------------------------|
| Control      | 2.56E+01 ±2.36E-02   | 7.30 ±2.85E-02       | 1.59E-07 ±2.63E-02 | 1.00 ±1.83E-02       | -4.78E-08 ±7.90E-03     |
| (w^{118})    |                      |                      |                    |                      |                          |
| well-fed     |                      |                      |                    |                      |                          |
| Starved      | 2.93E+01 ±5.85E-02   | 1.10E+01 ±7.62E-02   | 3.75 ±5.85E-02     | 7.44E-02 ±3.05E-03   | -1.13 ±1.76E-02         |
| mir-310s     | 2.17E+01 ±5.31E-03   | 2.88 ±4.64E-02       | -4.42 ±5.31E-03    | 2.14E+01 ±7.86E-02   | 1.33 ±1.60E-03          |
| (KT40/KT40)  |                      |                      |                    |                      |                          |
| well-fed     | 2.39E+01 ±6.43E-03   | 5.36 ±3.61E-02       | -1.94 ±6.43E-03    | 3.83 ±1.71E-02       | 5.83E-01 ±1.94E-03      |
| Starved      | 2.99E+01 ±3.09E-02   | 1.15E+01 ±3.29E-02   | -3.18E-07 ±3.09E-02| 1.00 ±2.16E-02       | 9.57E-08 ±9.31E-03      |
| Control      | 2.86E+01 ±5.96E-02   | 1.04E+01 ±7.70E-02   | -1.15 ±5.96E-02    | 2.21 ±8.97E-02       | 3.45E-01 ±1.79E-02      |
| (w^{118})    |                      |                      |                    |                      |                          |
| Starved      | 2.88E+01 ±4.78E-02   | 9.92 ±6.64E-02       | -1.62 ±4.78E-02    | 3.07 ±1.04E-01       | 4.88E-01 ±1.44E-02      |
| mir-310s     | 2.80E+01 ±4.38E-02   | 9.46 ±2.56E-02       | -2.07 ±2.56E-02    | 4.20 ±7.39E-02       | 6.24E-01 ±7.71E-03      |
| (KT40/KT40)  |                      |                      |                    |                      |                          |
| well-fed     | 2.67E+01 ±3.29E-02   | 8.33 ±3.47E-02       | 3.18E-07 ±3.29E-02 | 1.00 ±2.26E-02       | -9.57E-08 ±9.89E-03     |
| Starved      | 2.67E+01 ±4.36E-02   | 8.46 ±6.54E-02       | 1.27E-01 ±4.36E-02 | 9.16E-01 ±2.75E-02   | -3.83E-02 ±1.31E-02     |
| Control      | 3.62E+01 ±2.55E-01   | 1.73E+01 ±2.59E-01   | 8.99 ±2.55E-01     | 1.96E-03 ±3.78E-04   | -2.71 ±7.68E-02         |
| (w^{118})    |                      |                      |                    |                      |                          |
| Starved      | 3.62E+01 ±5.30E-01   | 1.77E+01 ±5.31E-01   | 9.39 ±5.30E-01     | 1.50E-03 ±6.56E-04   | -2.83 ±1.59E-01         |
| mir-310s     | 1.83E+01 ±1.11E-02   | 0.00 ±1.11E-02       |                   |                      |                          |
| (KT40/KT40)  |                      |                      |                    |                      |                          |
| well-fed     | 1.82E+01 ±4.88E-02   | 0.00 ±4.88E-02       |                   |                      |                          |
| Starved      | 1.89E+01 ±4.61E-02   | -6.36E-07 ±4.61E-02  |                   |                      |                          |
| Control      | 3.06E+01 ±1.02E-01   | 1.22E+01 ±1.02E-01   | 1.22E+01 ±1.02E-01 | 9.15E-05 ±1.02E-01   | -4.04 ±1.02E-01         |
| (w^{118})    |                      |                      |                    |                      |                          |
| well-fed -RT | 3.08E+01 ±1.01E-01   | 1.27E+01 ±1.01E-01   | 1.27E+01 ±1.01E-01 | 1.05E-04 ±1.01E-01   | -3.98 ±1.01E-01         |
| Starved -RT  | 3.06E+01 ±1.08E-01   | 1.20E+01 ±1.08E-01   | 1.20E+01 ±1.08E-01 | 1.39E-04 ±1.08E-01   | -3.86 ±1.08E-01         |
| mir-310s     | 2.99E+01 ±7.07E-01   | 1.17E+01 ±7.07E-01   | 1.17E+01 ±7.07E-01 | 9.03E-05 ±7.07E-01   | -4.04 ±7.07E-01         |
| (KT40/KT40)  |                      |                      |                    |                      |                          |
| well-fed -RT | 3.10E+01 ±1.08E-01   | 1.20E+01 ±1.08E-01   | 1.20E+01 ±1.08E-01 | 1.39E-04 ±1.08E-01   | -3.86 ±1.08E-01         |
| Starved -RT  | 3.10E+01 ±1.08E-01   | 1.20E+01 ±1.08E-01   | 1.20E+01 ±1.08E-01 | 1.39E-04 ±1.08E-01   | -3.86 ±1.08E-01         |

Plate 6
|                      | Sucb               | Rpl32        |
|----------------------|--------------------|--------------|
| **Control (w^{1118})** |                    |              |
| well-fed             | 2.38E+01 ±3.20E-02 | 1.84E+01 ±2.71E-02 |
| starved             | 2.43E+01 ±2.38E-02 | 1.81E+01 ±4.62E-02 |
| **mir-310s (KT40/KT40)** |                    |              |
| well-fed             | 2.39E+01 ±3.60E-02 | 1.86E+01 ±5.21E-02 |
| starved             | 2.43E+01 ±1.63E-02 | 1.83E+01 ±3.91E-02 |

| **Control (w^{1118})** |                    |              |
| well-fed             | 5.40 ±4.19E-02     | 1.27E-06 ±2.71E-02 |
| starved             | 6.23 ±5.19E-02     | -6.36E-07 ±4.62E-02 |
| **mir-310s (KT40/KT40)** |                    |              |
| well-fed             | 5.24 ±6.33E-02     | 6.36E-07 ±5.21E-02 |
| starved             | 5.99 ±4.23E-02     | -6.36E-07 ±3.91E-02 |

| **Control (w^{1118})** |                    |              |
| well-fed             | 1.11E-06 ±3.20E-02 | 1.20E+01 ±1.02E-01 |
| starved             | 8.30E-01 ±2.38E-02 | 1.27E+01 ±1.01E-01 |
| **mir-310s (KT40/KT40)** |                    |              |
| well-fed             | 5.95E-01 ±3.20E-02 | 1.20E+01 ±1.08E-01 |
| starved             | 6.62E-01 ±7.16E-03 | 1.17E+01 ±7.07E-01 |

| **Control (w^{1118})** |                    |              |
| well-fed             | 1.00 ±2.20E-02     | 1.22E+01 ±1.02E-01 |
| starved             | 5.62E-01 ±9.29E-03 | 1.27E+01 ±1.01E-01 |
| **mir-310s (KT40/KT40)** |                    |              |
| well-fed             | 1.12 ±2.80E-02     | 1.20E+01 ±1.08E-01 |
| starved             | 6.62E-01 ±7.46E-03 | 1.17E+01 ±7.07E-01 |

**No Reverse Transcriptase**

|                      |                    |
|----------------------|--------------------|
| **Control (w^{1118})** |                    |
| well-fed             | 3.06E+01 ±1.02E-01 |
| starved             | 3.08E+01 ±1.01E-01 |
| **mir-310s (KT40/KT40)** |                    |
| well-fed             | 3.06E+01 ±1.08E-01 |
| starved             | 2.99E+01 ±7.07E-01 |

| **Control (w^{1118})** |                    |
| well-fed             | 2.11E-05 ±1.45E-05 |
| starved             | 1.51E-05 ±1.03E-05 |
| **mir-310s (KT40/KT40)** |                    |
| well-fed             | 2.45E-05 ±1.81E-05 |
| starved             | 3.05E-04 ±1.43E-04 |

| **Control (w^{1118})** |                    |
| well-fed             | -3.68 ±3.08E-02    |
| starved             | -3.82 ±3.05E-02    |
| **mir-310s (KT40/KT40)** |                    |
| well-fed             | -3.61 ±3.24E-02    |
| starved             | -3.52 ±2.13E-01    |

a The relative mRNA levels were calculated by $2^{\Delta\Delta CT}$.

b Average (AVE) and standard error of the mean (SEM) values were calculated based on three replicates for each genotype/condition/gene value.

c Significance was calculated using two-tailed non-paired Student’s t-test.

Flies were fed with nutritionally rich or poor medium for 10 days before analysis.
Table S3, related to Figure S1. *mir-310s* mutants exhibit global defects associated with nutritional stress

| Genotype/Condition          | *Control* (w1118) | *mir-310s* (KT40/KT40) | *Control* (w1118) | *mir-310s* (KT40/KT40) |
|----------------------------|------------------|------------------------|------------------|------------------------|
| Phenotype                  | *Control*        | *mir-310s*             | *Control*        | *mir-310s*             |
|                            | (w1118) well-fed | (KT40/KT40) well-fed  | (w1118) starved  | (KT40/KT40) starved    |
|                            | (AVE±SEM)        | (AVE±SEM)              | (AVE±SEM)        | (AVE±SEM)              |
|                            | n=number of crops analyzed | n=number of crops analyzed | n=number of crops analyzed | n=number of crops analyzed |
| Crop diameter\(^a\):      | 0.65±0.05        | 0.85±0.04              | 0.44±0.05        | 0.44±0.04              |
| (in mm)                    | n=12             | n=10                   | n=10             | n=10                   |
| p\(^b\) Control well-fed = 0.007 | p\(^b\) Control well-fed = 1.00 | p\(^b\) Control well-fed = 0.007 | p\(^b\) Control well-fed = 0.007 |
| Lipid Accumulation:        | (3 days)         | (3 days)               | (10 days)        | (10 days)              |
| µg TAG equivalents per mg protein | 386.77±35.68     | 210.67±28.57           | 582.07±217.43   | 1581.0±202.03          |
| (AVE±SEM)                  | n=30             | n=20                   | n=30             | n=30                   |
| p\(^b\) Control well-fed = 0.008 | p\(^b\) Control well-fed = 0.04 | p\(^b\) Control well-fed = 0.04 | p\(^b\) Control well-fed = 0.0002 |
| Fecundity:                 | 16.48±1.76       | 6.03±0.4               | 1±0.01           | 0.11±0.04              |
| Eggs laid per fly per day | n=49             | n=50                   | p\(^b\) Control well-fed = 0.004 | p\(^b\) Control well-fed = 4.5E-01 |
| (AVE±SEM)                  | n=number of females analyzed | n=number of females analyzed | n=50             | n=50                   |
| Relative egg laying        | well-fed         | 1 day starved          | 2 day starved    | 3 day starved          | 4 day starved          |
| efficiency under starvation| 1±0.01           | 0.95±0.08              | 0.49±0.08        | 0.14±0.03              | 0.06±0.02              |
| *Control* (w1118)          | n=50             | p\(^b\) Control well-fed = 0.04 | p\(^b\) Control 1 day starved = 0.31±0.01 | p\(^b\) Control 1 day starved = 0.012±0.03 |
| n=50                       | 7.82E-04         | 5.48E-04               | 8.9E-03          | 2.22E-02               | 4.5E-01               |

\(^a\) Flies were fed with nutritionally rich and starvation medium for 10 days prior to analysis.

\(^b\) Maximum crop diameters were measured from bright field images using Adobe Photoshop software.

Three biological replicates were analyzed for each genotype/condition.

Significance was tested using two-tailed non-paired Student’s t-test.
Table S4, related to Figure 3. The mir-310s target Rab23, DHR96, and ttk in vitro

| 3’UTR Reporter | Control 3’UTR without mir-310s binding site | Rab23 3’UTR | DHR96 3’UTR | ttk 3’UTR | negative control short Dg 3’UTR without mir-310s binding sitea | positive control long Dg 3’UTR with mir-310s binding siteb |
|----------------|---------------------------------------------|-------------|-------------|-----------|-------------------------------------------------|---------------------------------------------|
| Luciferase Signal (Renilla/Firefly) | 7.76E-02 ±3.62E-03 | 2.41E-02 ±3.96E-03 | 3.75E-02 ±2.10E-03 | 3.60E-02 ±3.18E-03 | 9.16E-02 ±1.96E-03 | 2.09E-02 ±8.29E-04 |
| Relative Luciferase Signal | 1.00 ±4.67E-02 | 3.11E-01 ±5.10E-02 | 4.83E-01 ±2.71E-02 | 4.63E-01 ±4.10E-02 | 1.18 ±2.52E-02 | 2.69E-01 ±1.07E-02 |

Luciferase reporter assays were performed in three biological replicates for each gene.

Significance was tested using two-tailed non-paired Student’s t-test.

The short (a) and long (b) 3’UTRs of a confirmed mir-310s target gene, Dystroglycan (Dg) (YATSENKO et al. 2014), were used as negative and positive controls, respectively.
Table S5, related to Figure 2 and 3. Relative mRNA and miRNA expression levels

| Genotype/Condition | $C_{T}^{\text{Norm}}$ AVE±SEM | $C_{T}^{\text{Fold}}$ AVE±SEM | $\Delta C_{T}$ AVE±SEM | $\Delta\Delta C_{T}$ AVE±SEM | Relative Rab23 mRNA level$^{*}$ AVE±SEM |
|--------------------|-------------------------------|-------------------------------|----------------------|-------------------------|---------------------------------|
| Control (w1118)    | 2.42E±01 ±2.7E-01              | 1.85E±01 ±1.97E-01            | 5.71 ±8.18E-02       | 0.00                    | 1.00 ±5.18E-02                  |
| mir-310s (KT40/KT40)| 2.41E±01 ±1.04E-01            | 1.9E±01 ±5.34E-02             | 5.06 ±6.22E-02       | -6.48E-01               | 1.57 ±7.08E-02                  |
| Control (w1118)    | 2.8E±01 ±3.1E-01               | 1.86E±01 ±1.21E-01            | 9.32 ±7.23E-02       | 3.61 ±2.67E-01          | 8.17E-02 ±1.4E-02               |
| mir-310s (KT40/KT40)| 2.59E±01 ±1.98E-01            | 1.87E±01 ±9.29E-02            | 7.2 ±1.15E-02        | 1.49 ±1.52E-01          | 3.56E-01 ±3.47E-02               |

| Genotype/Condition | $C_{T}^{\text{Norm}}$ AVE±SEM | $C_{T}^{\text{Fold}}$ AVE±SEM | $\Delta C_{T}$ AVE±SEM | $\Delta\Delta C_{T}$ AVE±SEM | Relative DHR96 mRNA level AVE±SEM |
|--------------------|-------------------------------|-------------------------------|----------------------|-------------------------|---------------------------------|
| Control (w1118)    | 2.66E±01 ±1.87E-01              | 1.83E±01 ±1.34E-01            | 8.24 ±6.43E-02       | 0.00                    | 1.00 ±3.23E-02                  |
| mir-310s (KT40/KT40)| 2.66E±01 ±1.52E-01            | 1.9E±01 ±5.72E-02             | 7.61 ±8.62E-02       | -6.35E-01               | 1.55 ±9.11E-02                  |
| Control (w1118)    | 2.85E±01 ±1.14E-01            | 1.86E±01 ±1.14E-01            | 9.93 ±9.36E-02       | 1.69 ±9.36E-02          | 3.12E-01 ±1.43E-02               |
| mir-310s (KT40/KT40)| 2.75E±01 ±1.11E-01            | 1.86E±01 ±5.79E-02            | 8.87 ±6.06E-02       | 6.28E-01                | 6.47E-01 ±1.53E-02               |

| Genotype/Condition | $C_{T}^{\text{Norm}}$ AVE±SEM | $C_{T}^{\text{Fold}}$ AVE±SEM | $\Delta C_{T}$ AVE±SEM | $\Delta\Delta C_{T}$ AVE±SEM | Relative tub mRNA level AVE±SEM |
|--------------------|-------------------------------|-------------------------------|----------------------|-------------------------|---------------------------------|
| Control (w1118)    | 2.56E±01 ±2.48E-01              | 1.89E±01 ±2.06E-01            | 6.67 ±5.53E-02       | 0.00                    | 1.00 ±4.04E-02                  |
| mir-310s (KT40/KT40)| 2.64E±01 ±9.0E-02             | 1.97E±01 ±2.12E-01            | 6.66 ±1.22E-01       | -4.0E-03                | 1.002 ±8.94E-02                 |
| Control (w1118)    | 2.69E±01 ±1.18E-01            | 1.9E±01 ±1.08E-01             | 7.82 ±3.42E-01       | 1.16 ±3.42E-01          | 0.45 ±4.2E-02                    |
| mir-310s (KT40/KT40)| 2.64E±01 ±1.13E-01            | 1.93E±01 ±1.53E-01            | 7.17 ±1.39E-01       | 5.06E-01                | 0.70 ±9.93E-02                   |

| Genotype/Condition | $C_{T}^{\text{Norm}}$ AVE±SEM | $C_{T}^{\text{Fold}}$ AVE±SEM | $\Delta C_{T}$ AVE±SEM | $\Delta\Delta C_{T}$ AVE±SEM | Relative mir-310 level AVE±SEM |
|--------------------|-------------------------------|-------------------------------|----------------------|-------------------------|---------------------------------|
| Control (w1118)    | 2.54E±01 ±2.26E-00              | 1.05E±01 ±2.26E-00            | 1.49E±01 ±4.45E-02   | 0.00                    | 1.00 ±5.86E-02                  |
| Control (w1118)    | 2.42E±01 ±2.01E-00              | 1.05E±01 ±2.01E-00            | 1.43E±01 ±6.58E-02   | -6.14E-01               | 1.54E-01 ±1.12E-01               |

| Genotype/Condition | $C_{T}^{\text{Norm}}$ AVE±SEM | $C_{T}^{\text{Fold}}$ AVE±SEM | $\Delta C_{T}$ AVE±SEM | $\Delta\Delta C_{T}$ AVE±SEM | Relative mir-312 level AVE±SEM |
|--------------------|-------------------------------|-------------------------------|----------------------|-------------------------|---------------------------------|
| Control (w1118)    | 2.55E±01 ±2.26E-00              | 9.48E±01 ±1.60E-00            | 1.60E±01 ±6.67E-01   | 0.00                    | 1.00 ±5.62E-02                  |
| Control (w1118)    | 2.86E±01 ±2.23E-00              | 1.13E±01 ±1.31E-00            | 1.54E±01 ±2.54E-01   | 5.27E-01                | 1.49 ±2.33E-01                   |

$^a$ The relative mRNA levels were calculated by $2^{-\Delta C_{T}}$.
$^b$ Average (AVE) and standard error of the mean (SEM) values were calculated using at least three biological replicates for each genotype and condition.
$^c$ Significance was tested using two-tailed non-paired Student’s t-test.
Flies were fed with nutritionally rich and poor medium for 10 days prior analysis.
Table S6, related to Figure 4. Rab23 is upregulated at the germarial niche upon *mir-310s* loss

| Genotype/ Condition | Rab23-expressing CpC percentage | AVE±SEM | negative | positive | high |
|---------------------|-------------------------------|---------|----------|-----------|------|
|                     |                               |         | low      | high      |      |
| *w*^1118^; Rab23::YFP::4xmyc | well-fed                       | n=6     | 17.75±2.41% | 36.33±5.34% | 45.92±6.19% |
| *mir-310s*; Rab23::YFP::4xmyc | well-fed                       | n=6     | 4.46±3.1%   | 51.19±9.4%  | 44.35±10.95% |
| *w*^1118^; Rab23::YFP::4xmyc | starved                        | n=6     | 6.94±3.47%  | 72.22±4.36% | 20.83±4.08% |
| *mir-310s*; Rab23::YFP::4xmyc | starved                        | n=6     | 7.54±3.71%  | 35.19±3.09% | 57.28±4.7% |

a Averages and the standard errors of the means were calculated using five replicates.

Significances between the percentages of the cap cells (CpCs) that differentially express Rab23 protein: Rab23 negative CpCs under well-fed condition and the CpCs that have high Rab23 expression under starvation condition were calculated using a two tailed Student’s t-test.

In order to analyze the significance between the frequencies of CpCs that differentially express Rab23 protein [negative or positive (high or low)] in control and *mir-310s* mutant germaria under well-fed and starved conditions, two-way tables and chi-squared test with 6 degrees of freedom were used. Chi-square value is 11.311 and p value is 0.079227.
Table S7, related to Figure 4. Upon mir-310s loss or Rab23 overexpression, the number of Hh-positive speckles in the germarium increases

| Genotype                        | number Hh speckles AVE±SEM | well-fed   | starved |
|---------------------------------|-----------------------------|------------|---------|
| **Control**                     |                             |            |         |
| (w1118/Oregon-R-C)              |                             | 92.67±3.66 | 55.11±8.62 |
|                                 |                             | n=9        |         |
| **mir-310s**                    |                             | 198.67±17.53 | 169.33±6.09 |
| (KT40/KT40)                     |                             | n=9        |         |
|                                 |                             | p<sub>Control well-fed</sub> = 7.25E-05 | p<sub>Control starved</sub> = 9.04E-09 |
| **bab1>Rab23**                  |                             | 260.0±26.86 | 198.89±11.96 |
| (bab1-Gal4/UAS-Rab23)           |                             | n=9        |         |
|                                 |                             | p<sub>Control well-fed</sub> = 2.41E-05 | p<sub>bab1>Rab23 starved</sub> = 3.89E-08 |

Confocal images were analyzed using the particle analyzer tool from ImageJ software to quantify Hedgehog (Hh) speckle numbers.

p-values were calculated using two-tailed non-paired Student’s t-test.
| CG number | Gene name       | Table S8, related to Figure 4. Rab23 co-immunoprecipitated proteins |
|-----------|----------------|--------------------------------------------------------------------|
| CG2108    | Rab23          | CG5641                 |
| CG7920    | CG7920         | CG5641                 |
| CG2152    | Pemt           | CG8053                 |
| CG4916    | mec31B         | CG6341                 |
| CG7445    | fln            | CG4008                 |
| CG30395   | CG30395        | CG4170                 |
| CG6821    | Lsp1gamma      | CG4666                 |
| CG6803    | Mf             | CG10279                |
| CG8867    | Jon25Bi        | CG1469                 |
| CG9769    | elf3-S5-1      | CG13849                |
| CG5887    | desat1         | CG6987                 |
| CG5654    | yps            | CG8189                 |
| CG7113    | scu            | CG4913                 |
| CG4153    | elf2-beta      | CG4912                 |
| CG4466    | Hsp27          | CG6258                 |
| CG1742    | Mgstl          | CG8427                 |
| CG16765   | ps             | CG10851                |
| CG7178    | wupA           | CG3972                 |
| CG11844   | vig2;fdy       | CG14999                |
| CG5330    | Nap1           | CG6617                 |
| CG2229    | Jon99Fii       | CG4003                 |
| CG4769    | CG4769         | CG17136                |
| CG10306   | CG10306        | CG31362                |
| CG3800    | CG3800         | CG14813                |
| CG4533    | l(2)ectl       | CG10206                |
| CG4183    | Hsp26          | CG5313                 |
| CG18811   | Capr           | CG5352                 |
| CG8308    | alphatub67C    | CG32701                |
| CG1633    | Jfrrac1        | CG8231                 |
| CG9641    | CG9641         | CG4376                 |
| CG45077   | fau            | CG8142                 |
| CG34069   | mt:ColII       | CG4978                 |
| CG5422    | Rox8           | CG4661                 |
| CG8871    | Jon25Bii       | CG13240                |
| CG5885    | BEST:ck01296  | CG11835                |
| CG13425   | bl             | CG45076                |
| CG5258    | NHP2           | CG7172                 |
| CG10922   | La             | CG7436                 |
| CG10578   | DnaJ-1         | CG6693                 |
| CG10849   | Sc2            | CG9306                 |
| CG6543    | CG6543         | CG7917                 |
| CG4302    | BEST:GH09395  | CG15092                |
|           |                | CG8977                 |
|           |                | CG13887                |
|           |                | CG7637                 |
|           |                | CG18067                |
|           |                | CG8844                 |
|           |                | CG17686                |
|           |                | CG5289                 |
|           |                | CG5047                 |
|           |                | CG4799                 |
|           |                | CG11107                |
|           |                | CG5374                 |
|           |                | CG4422                 |
|           |                | CG18591                |
|           |                | CG8715                 |
|           |                | CG4082                 |
|           |                | CG2216                 |
|           |                | CG12203                |
|           |                | CG10628                |
|           |                | CG5329                 |
|           |                | CG5167                 |
|           |                | CG12306                |
|           |                | CG4729                 |
|           |                | CG6519                 |
|           |                | CG30185                |
|           |                | CG7182                 |
|           |                | CG17566                |
|           |                | CG11999                |
|           |                | CG16725                |
|           |                | CG7280                 |
|           |                | CG3446                 |
|           |                | CG12400                |
|           |                | CG4553                 |
|           |                | CG8322                 |
|           |                | CG3039                 |
|           |                | CG6094                 |
|           |                | CG10097                |
|           |                | CG1489                 |
|           |                | CG14207                |
|           |                | CG17611                |
|           |                | CG3333                 |
|           |                | CG7409                 |
|           |                | CG3944                 |
|           |                | CG30008                |
|           |                | CG5371                 |
|           |                | CG2367                 |
|           |                | CG4824                 |
|           |                | CG5903                 |
|           |                | CG15481                |
|           |                | CG14476                |
|           |                | CG3436                 |
|           |                | CG31249                |
|           |                | CG6746                 |
|           |                | CG7581                 |
|           |                | CG7378                 |
|           |                | CG8905                 |
|           |                | CG6013                 |
|           |                | CG1616                 |
|           |                | CG1938                 |
|           |                | CG4634                 |
|           |                | CG13162                |
|           |                | CG5703                 |
|           |                | CG31523                |
|           |                | CG9155                 |
|           |                | CG8258                 |
|           |                | CG30176                |
|           |                | CG8947                 |
|           |                | CG3710                 |
|           |                | CG3606                 |
|           |                | CG1249                 |
|           |                | CG13163                |
|           |                | CG3683                 |
|           |                | CG12984                |
|           |                | CG8547                 |
|           |                | CG8542                 |
|           |                | CG7033                 |
|           |                | CG4206                 |
|           |                | CG12163                |
|           |                | CG3564                 |
|           |                | CG10833                |
|           |                | CG5826                 |
|           |                | CG8190                 |
|           |                | CG5183                 |
|           |                | CG7006                 |
|           |                | CG12357                |
|           |                | CG4274                 |
|           |                | CG7830                 |

21
| Gene ID  | Description      |
|----------|-------------------|
| CG16912  | CG16912           |
| CG3508   | Bcd              |
| CG3416   | Mov34            |
| CG7483   | elf4AIII         |
| CG17437  | wds              |
| CG4020   | CG4020           |
| CG9548   | CG9548           |
| CG18444  | alphaTry         |
| CG1101   | Refl             |
| CG10297  | Acp65Aa          |
| CG5000   | mspse            |
| CG3420   | CG3420           |
| CG14309  | CG14309          |
| CG9987   | CG9987           |
| CG7123   | LanB1            |
| CG1751   | Spase25          |
| CG8680   | CG8680           |
| CG6137   | aub              |
| CG3422   | CG3422           |
| CG10469  | CG10469          |
| CG7619   | CG7619           |
| CG1828   | CG1828           |
| CG34026  | CG34026          |
| CG3359   | mfas             |
| CG7361   | CG7361           |
| CG9054   | Ddx1             |
| CG8351   | CG8351           |
| CG16904  | CG16904          |
| CG11804  | CG11804          |
| CG9302   | CG9302           |
| CG7697   | CG7697           |
| CG9172   | CG9172           |
| CG9383   | CG9383           |
| CG10045  | CG10045          |
| CG7488   | CG7488           |
| CG4760   | CG4760           |
| CG1453   | CG1453           |
| CG6782   | CG6782           |
| CG7008   | CG7008           |
| CG11876  | CG11876          |
| CG4463   | CG4463           |
| CG4279   | CG4279           |
| CG11989  | CG11989          |
| CG5864   | CG5864           |
| CG44255  | CG44255          |
| CG10212  | CG10212          |
| CG10470  | CG10470          |
| CG2910   | nito             |
| CG15735  | CG15735          |
| CG1877   | lin19            |
| CG8749   | snRNP-U1-70K     |
| CG5548   | CG5548           |
| CG8711   | Cul-4            |
| CG16983  | skpA             |
| CG18559  | Cyp309a2         |
| CG7946   | CG7946           |
| CG3845   | NAT1             |
| CG11429  | CG11429          |
| CG33104  | eca,p24-2        |
| CG2014   | CG2014           |
| CG5555   | CG5555           |
| CG9741   | Dhdod            |
| CG3424   | path             |
| CG10687  | Aats-asn         |
| CG2621   | sgg              |
| CG13091  | CG13091          |
| CG42807  | CG6183           |
| CG3917   | Grip84           |
| CG3909   | CG3909           |
| CG3664   | Rab5             |
| CG3059   | NTPase           |
| CG15877  | CG15877          |
| CG32441  | CG32441          |
| CG6416   | Zasp66           |
| CG1548   | cathD            |
| CG8409   | Su(var)205       |
| CG13277  | LSm7             |
| CG10203  | x16              |
| CG4115   | CG4115           |
| CG13570  | spag             |
| CG12908  | Ndg              |
| CG11785  | CG11785          |
| CG15531  | CG15531          |
| CG6249   | Csl4             |
| CG8827   | A nec            |
| CG3200   | Reg-2            |
| CG1703   | CG1703           |
| CG4447   | CG4447           |
| CG11837  | CG11837          |
| CG7359   | Sec22            |
| CG5670   | Atpalpha         |
| CG10360  | ref(2)P          |
| CG2604   | CG2604           |
| CG5252   | Ranbp9           |
| CG30149  | rig              |
| CG6235   | tws              |
| CG3678   | CG17556          |
| CG10210  | CG10210          |
| CG8548   | Kap-alpha1       |
| CG3068   | aur              |
| CG2175   | CG2175           |
| CG6375   | pit              |
| CG3295   | CG3295           |
| CG9018   | CG9018           |
| CG3959   | peio             |
| CG7979   | CG7979           |
| CG14224  | Ubqn             |
| CG11092  | Nup93-1          |
| CG6866   | loqs             |
| CG1119   | Gnf1             |
| CG8625   | Iswi             |
| CG9128   | Sac1             |
| CG3815   | CG3815           |
| CG4051   | egl              |
| CG34074  | mt-ColI          |
| CG1091   | CG1091           |
| CG13935  | Cpr62Bb          |
| CG3299   | Vinc             |
| CG8397   | CG8397           |
| CG2867   | Prat             |
| CG11015  | CoVb             |
| CG9889   | yellow-d         |
| CG2071   | Ser6             |
| CG3582   | U2af58           |
| CG3561   | Dbp21E2          |
| CG8648   | Fen1             |
| CG7833   | Orc5             |
| CG33141  | sns              |
| CG7288   | CG7288           |
| CG2031   | Hpr1             |
| CG1307   | CG1307           |
| CG9749   | Abi              |
| CG5272   | gnu              |
| CG10159  | BEAP-32          |
| CG31368  | CG31368          |
| CG11137  | CG11137          |
| CG3071   | Eg-25E8.3        |
| CG14788  | ns3              |
| CG4088   | lat              |
| CG7109   | mts              |
| CG3056   | sxx              |
| CG9159   | CG9159           |
| CG31717  | CG31717          |
| CG18347  | CG18347          |
| CG4038   | CG4038           |
| CG10498  | edc2c            |
| CG13472  | CG13472          |
| CG6841   | CG6841           |
| CG9350   | CG9350           |
| CG10472  | CG10472          |
| CG6948   | Clic             |
| CG12000  | Probeta7         |
| CG1179   | LysB-LysD-LysA-LysE |
| CG11777  | CG11777          |
| CG1685   | pen              |
| CG33129  | CG6089           |
| CG33503  | Cyp12d1-d        |
| CG4039   | Mcm6             |
| CG9547   | CG9547           |
| CG10333  | CG10333          |
| CG9441   | Pu               |
| CG3157   | gammaTub23 C    |
| CG5001   | CG5001           |
| CG5193   | ThiIB            |
| CG18124  | mTTF             |
| CG7929   | ocn              |
| CG12128  | CG12128          |
| CG3320   | Rab1             |
| CG1401   | CG1401           |
| CG3412   | slmb             |
| CG15433  | Elp3             |
| CG4152   | l(2)35Df         |
| CG3501   | CG3501           |
| CG11397  | glu              |
| CG9253   | CG9253           |
| CG4365   | CG4365           |
| CG17454  | CG17454          |
| CG7970   | CG7970           |
| CG1406   | U2A              |
| CG5999   | msi              |
| CG3625   | CG3625           |
| CG5358   | Art4             |
| CG8571   | smid             |
| CG11583  | CG11583          |
| CG10326  | CG10326          |
| CG17018  | CG17018          |
| CG8553   | SelD             |
Co-immunoprecipitated protein hits were filtered for 5-fold enrichment in the tagged Rab23 sample (w¹¹¹⁸; Rab23::YFP::4xmyc) compared to control (w¹¹¹⁸), resulting in 821 unique proteins.

COPI-associated proteins are highlighted.
Table S9, related to Figures 5 and 6. The frequencies of the analyzed ovarian phenotypes

| Genotype | Phenotype | Disorganized gerarium architecture at region 2A/B | Abnormal egg chamber encapsulation | Multilayered stalk | Persisting FasIII expression | Multilayered follicular epithelium |
|----------|-----------|-----------------------------------------------|-----------------------------------|--------------------|-------------------------------|----------------------------------|
| Control  | (w1118/Oregon-R-C) | 26.7% n=30 | 0% n=20 | 5% n=20 | 0% n=35 | well-fed<sup>a</sup> 15% n=20 | well-fed<sup>a</sup> 5% n=20 | starved<sup>a</sup> 0% n=20 | starved<sup>a</sup> 0% n=20 |
| mir-310s (KT40/KT40) | 86.7% n=30 | 35% n=20 | 75% n=20 | 44.4% n=35 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sub> Control</sub>=0.0001 | well-fed<sup>a</sup> 45% n=20 | well-fed<sup>a</sup> 5% n=20 | starved<sup>a</sup> 0% n=20 | starved<sup>a</sup> 0% n=20 |
| mir-310s (w1<sup>1118</sup>; Df(2R)mir-310-311-312-313 FRT42D) | 66.7% n=30 | 5% n=20 | 65% n=20 | 54.2% n=35 | p<sub>C<sup>C</sup> Control</sub>=0.0002 | p<sub>C<sup>C</sup> Control</sub>=0.311 | p<sub>C<sup>C</sub> Control</sub>=0.0011 | 50% n=20 | 50% n=20 | p<sub>C<sup>C</sub> Control</sub>=0.018 |
| mir-310s/Df(2R)Exel607 0, P[w[+mC]=XP-U]<sub>FRT42D</sub> | 80% n=30 | 40% n=20 | 70% n=20 | 59.1% n=35 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sub> Control</sub>=0.0001 | 70% n=20 | 70% n=20 | p<sub>C<sup>C</sub> Control</sub>=0.0001 |
| babs1>hh (tub-Gal80<sup>0</sup>/++; babs1-Gal4/UAS-hh) | 100% n=30 | 95% n=20 | 100% n=20 | 48% n=35 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sub> Control</sub>=0.0001 | well-fed<sup>a</sup> 100% n=20 | well-fed<sup>a</sup> 100% n=20 | starved<sup>a</sup> 50% n=20 | starved<sup>a</sup> 50% n=20 |
| babs1>Rab23 (babs1-Gal4/UAS-Rab23) | 76.7% n=30 | 35% n=20 | 70% n=20 | 52.2% n=35 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sub> Control</sub>=0.0001 | 35% n=20 | 35% n=20 | p<sub>C<sup>C</sub> Control</sub>=0.144 |
| Rescue mir-310s (KT40/KT40; attB2 mir-310s res long 2/+) | 33.3% n=30 | 5% n=20 | 30% n=20 | 16% n=35 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sup> Control</sub>=0.0001 | p<sub>C<sup>C</sub> Control</sub>=0.0001 | 35% n=20 | 35% n=20 | p<sub>C<sup>C</sub> Control</sub>=0.027 |
| mir-310s: babs1>hh RNAi (KT40/KT40; babs1-Gal4/ UAS-hh-RNAi) | 50% n=30 | 12% n=20 | 20% n=20 | 28.6% n=35 | p<sub>C<sup>C</sup> Control</sub>=0.015 | p<sub>C<sup>C</sub> Control</sub>=0.008 | p<sub>C<sup>C</sub> Control</sub>=0.0011 | 15% n=20 | 15% n=20 | p<sub>C<sup>C</sub> Control</sub>=0.0001 |
| mir-310s: babs1>Rab23 RNAi (KT40/KT40; babs1-Gal4/ UAS-Rab23-RNAi) | 46.7% n=30 | 20% n=20 | 20% n=20 | 25% n=35 | p<sub>C<sup>C</sub> Control</sub>=0.0007 | p<sub>C<sup>C</sub> Control</sub>=0.0168 | p<sub>C<sup>C</sub> Control</sub>=0.0001 | 40% n=20 | 40% n=20 | p<sub>C<sup>C</sub> Control</sub>=0.057 |

a Flies were kept on nutritionally rich or poor medium for 7 days prior to analysis.

b tub-Gal80<sup>0</sup>/++; babs1-Gal4/UAS-hh flies were kept for 3 days at restrictive temperature (29°C).

Occurrences of the listed phenotypes per ovariole are indicated as percentages.

Significance was tested using Pearson’s chi-Square test and IBM SPSS Statistics software.
Table S10, related to Figure 6. The high mitotic activity in mir-310s mutant egg chambers is rescued by downregulating Rab23 or Hh levels

| Genotype | Number of PH3+ follicle cells (AVE±SEM) | n=number of stage 2 egg chambers analyzed |
|----------|----------------------------------------|----------------------------------------|
|          | well-fed (7 days)                       | Starved (7 days)                        |
| Control  | 4.17±0.25 n=30                         | 0.20±0.09 n=30                         |
| (w[1118]/Oregon-R-C) |                                   |                                        |
| bab1>hh RNAi$^a$ (tub-Gal80$^{+/+}$; bab1-Gal4/UAS-hh-RNAi) | 2.00±0.34 n=30 | 0.27±0.12 n=15 | $^{p_{Control_{well-fed}}=1.6E-05}$ | $^{p_{Control_{starved}}=0.378}$ |
| bab1>Rab23 RNAi$^a$ (tub-Gal80$^{+/+}$; bab1-Gal4/UAS-Rab23-RNAi) | 2.4±0.33 n=30 | 0.20±0.11 n=15 | $^{p_{Control_{well-fed}}=1.04E-04}$ | $^{p_{Control_{starved}}=0.50}$ |
| bab1>hh (tub-Gal80$^{+/+}$; bab1-Gal4/UAS-hh) | 8.4±0.68$^b$ n=30 | 1.07±0.23$^b$ n=15 | $^{p_{Control_{well-fed}}<0.00001}$ | $^{p_{Control_{starved}}=0.006}$ |
| bab1>Rab23 (tub-Gal80$^{+/+}$; bab1-Gal4/UAS-Rab23) | 6.37±0.68 n=30 | 1.13±0.29 n=15 | $^{p_{Control_{well-fed}}=0.0036}$ | $^{p_{Control_{starved}}=0.007}$ |
| mir-310s/ Df6070 (w[1118]; KT40/Df(2R)Exel6070, P[w/+mC]=XP-U{Exel6070}) | 5.3±0.38 n=30 | 0.70±0.16 n=30 | $^{p_{Control_{well-fed}}=0.0233}$ | $^{p_{Control_{starved}}=0.011}$ |
| mir-310s (KT40/KT40) | 5.63±0.51 n=30 | 0.8±0.19 n=30 | $^{p_{Control_{well-fed}}=0.0222}$ | $^{p_{Control_{starved}}=0.015}$ |
| Rescue mir-310s (KT40/KT40; attB2 mir-310s res long 2+/+) | 4.23±0.27 n=30 | 0.13±0.29 n=15 | $^{p_{KT40/Df6070}=0.0367}$ | $^{p_{KT40/Df6070}=0.0197}$ |
| mir-310s; bab1>hh RNAi (KT40/KT40; bab1-Gal4/UAS-hh-RNAi) | 4.03±0.26 n=30 | 0.4±0.13 n=15 | $^{p_{KT40/Df6070}=0.011}$ | $^{p_{KT40/Df6070}=0.1075}$ |
| mir-310s; bab1>Rab23 RNAi (KT40/KT40; bab1-Gal4/UAS-Rab23-RNAi) | 4.2±0.27 n=30 | 0.33±0.13 n=15 | $^{p_{KT40/Df6070}=0.0367}$ | $^{p_{KT40/Df6070}=0.0735}$ |

Significance was tested using Mann-Whitney U test and z statistic.

$^a$ Flies were kept at restrictive temperature (29°C) for 7 days.

$^b$ Flies were kept at restrictive temperature (29°C) for 3 days.
| 3'UTR Luciferase reporter cloning<sup>a</sup> | Rab23 | NotI | Forward | GCAAGCGGCCGCTTTTTGCATAGAATGCGAGCAGC |
|---------------------------------------------|-------|------|----------|--------------------------------------|
| XhoI | Reverse | GCAACTCGAGGACCCGCGAATCAAAATAATAAACAAG |
| ttk | XhoI | Forward | GCAACTCGAGAAGGCGCAATCCAAAGATGCGAG |
| NotI | Reverse | GCAAGCGGCCGCGGAGAAATTGCTGAAGGTTT |
| DHR96 | XhoI | Forward | GCAACTCGAGTGTCGTTTTTTATGTTGCTCGTGT |
| NotI | Reverse | GCAAGCGGCCGCTTTTTGGCATGACCAACCCAC |

| Rab23 | qRT-PCR | Forward |
|-------|---------|---------|
| DHR96 | AGCTGGCCATTAAAGTGTTGCTATT |
| ttk | GATCTCGAGTCTGCTCGTCAGAG |
| Rpl32 | CCTAGGATCCTCGGCTCCAAGAAG |
| Act88F | CAGCTGCAATAGCTTTGGGTTGTTG |
| ade2 | CGAAAGATCAGAAAAGCTCAACAG |
| ade3 | CGGCGAGACGAGGAGGAGGAGG |
| Arrl | CCTGCTGCTGGGCTCCCTC |
| CG3699 | CAGGCACGCGCCAGGAGAAG |
| CG3902 | CACGCGGCTCCAGAGTATG |
| CG3999 | GAGGCTGCTGGAGCTGGAAAG |
| CG9914 | CTGGGCAGCCAGGAGGAGT |
| CG11089 | CCGCGAGATCCACAGATTTAG |
| CG15369 | CCGTGACCAAAAGCTCCT |
| CG16884 | CTGGGCAGCCAGGAGGAGT |
| CG30360 | CACGCGGCTCCAGAGTATG |
| CG31233 | CCGCCACGCGCCAGGAGAAG |
| Cpr62Bc | CGTGCTGCTGGAGCTGGAAAG |
| Cpr72Ec | CGGCGAGACGAGGAGGAGGAGG |
| Cpr100A | CTGGGCAGCCAGGAGGAGT |

<sup>a</sup> Primer sequences for luciferase reporter cloning and qRT-PCR.
| Gene     | Forward                        | Reverse                        |
|---------|--------------------------------|--------------------------------|
| Gal     | CCAGACGCTTTAGCGGGATTCA         | CCGGTGGCCTACCCACTAAGTA         |
| Gasp    | CTCGCCGGTTCCAGCAGTTCC          | CTCGCCGGTTACGGCATCTTCC         |
| GstD4   | TCCCCAGCACACCTTTCC             | CTTTGCCGAATTTGGTACGGTAG        |
| Lsp1beta| CCCGCCACGCACTGTCT              | CGCACGTTCAAGGCTAGAAGCA        |
| Lsp2    | TGCCCAACCAATGAGTACGGTT         | CGGGGCTGCTGCTGCTGAGT          |
| LvpH    | CGACTTGAATATGGGGGGAGCAGC       | ACGGCATTGCGGAACTCTTGA         |
| Mgstl   | GATGTCCCCCAAGCTGAAGGTCT        | GAGGAAGAAGGAGAGGAGATGGT       |
| mus209  | ACATCGACAGCTGACTTGGGT          | GCCGGTGACGCTGACATTG          |
| Obp44a  | TGCTCGCTGAGAAGAACTGT          | TGGCAGATACCCACATTTGAGC        |
| Obp56a  | CGCCTCAAGGTAGTACGGATTGC       | CGGAATCCACATTTGGCAAGCA        |
| Obp56e  | TGCCTCAAGCTTTTGGGAAATC        | TTGCCGCTGCTTAACTTTTTTGGG      |
| Obp99b  | CTCCCTCGCTGCGTGAACCT          | TCACCATACCATACCCAGCAC         |
| obst-A  | CATCCCAAGGACTGCGAAGA          | ATCGTGTAAGATCTCGTCAAGC        |
| pro-PO-A1| GGCAGTCCACGTCCCTCCAG         | CAGCAGAATAACCGCACTTA          |
| Suchb   | TGGGCTGATCTGCCTGCGTGTAAC      | CGGCAGTTTTCGGTTGTGTT         |

For cloning, cutting sites for indicated restriction enzymes were added to 5’ end of the designed primers.

All primers were designed using Lasergene Software.
File S1. Supplemental Experimental Procedures

SILAC labeling and MS/MS Analysis

Heavy amino acid-labeled (Lys-8, Lys-13C615N2, Cambridge Isotope Laboratories, Inc.) yeast and flies were cultivated as published (SURY et al. 2010). Lysine auxotrophic S. cerevisiae strain SUB62 (kindly provided by Matthias Selbach) was precultured 1:1000 for 24 hours and then inoculated for 1:100 and incubated for another 24 hours in defined, labeling medium before harvesting. Prior to feeding of Drosophila, incorporation of Lys-8 to yeast cells was measured by mass spectrometry and almost complete incorporation (>95%) was achieved. We used w1118 stock as the control strain. Control flies were grown with light-labeled (Lys-0, Lys-12C614N2, Sigma) and mir-310s mutant (KT40/KT40) flies with heavy-labeled yeast (Lys-8). In parallel, as a replicate experiment the reverse labeling was done, where control flies were fed with heavy and mir-310s mutant flies were fed with light-labeled yeast. Hatched flies were kept on the same medium with labeled yeast pellet for 3 days before harvesting. For sample preparation, 10 female flies were snap frozen in liquid nitrogen and homogenized in 100µl RIPA buffer (SURY et al. 2010) supplemented with 1X Protease inhibitor cocktail (Thermo). Total protein amounts were quantified using Bradford Reagent (Sigma). Samples containing 25µg of total protein from each labeling-genotype experiment were used for the analysis.

Proteins were separated by one-dimensional SDS-PAGE (4%–12% NuPAGE Bis-Tris Gel, Invitrogen) and stained with Coomassie Blue G-250 (Fluka). The complete gel lanes were cut into 23 equally sized slices. Proteins were digested as described previously (SHEVCHENKO et al. 2006). Briefly, proteins were reduced with 10 mM DTT for 50 min at 50°C, afterwards alkylated with 55
mM iodoacetamide for 20 min at 26°C. In-gel digestion was performed with Lys-C (Roche Applied Science) overnight. Extracted peptides from gel slices were loaded onto the in-house packed C18 trap column (ReproSil-Pur 120 C18-AQ, 5 μm, Dr. Maisch GmbH; 20 x 0.100 mm) at a flow rate of 5 μl/min loading buffer (2% acetonitrile, 0.1% formic acid). Peptides were separated on the analytical column (ReproSil-Pur 120 C18-AQ, 3 μm, Dr. Maisch GmbH; 200 x 0.050 mm, packed in-house into a PF360-75-15-N picofrit capillary, New Objective) with a 90 min linear gradient from 5% to 40% acetonitrile containing 0.1% formic acid at a flow rate of 300 nl/min using nanoflow liquid chromatography system (EASY n-LC 1000, Thermo Scientific) coupled to hybrid quadrupole-Orbitrap (Q Exactive, Thermo Scientific). The mass spectrometer was operated in data-dependent acquisition mode where survey scans acquired from m/z 350-1600 in the Orbitrap at resolution settings of 70,000 FWHM at m/z 200 at a target value of 1 x 10E6. Up to 15 most abundant precursor ions with charge states 2+ or more were sequentially isolated and fragmented with higher collision-induced dissociation (HCD) with normalized collision energy of 28. Dynamic exclusion was set to 18 s to avoid repeating the sequencing of the peptides.

The generated raw Mass Spectrometry files were analyzed with MaxQuant software (version 1.3.0.5, using Andromeda search engine) (COX AND MANN 2008) against UniProtKB D. melanogaster database containing 18826 entries (downloaded in April 2013) and Flybase D. melanogaster database (release 6.02) supplemented with common contaminants and concatenated with the reverse sequences of all entries. The following Andromeda search parameters were set: carboxymethylation of cysteines as a fixed modification, oxidation of methionine and N-terminal
acetylation as a variable modification; and Lys-C specificity with no proline restriction and up to two missed cleavages. The MS survey scan mass tolerance was 7 ppm and for MS/MS 20 ppm. For protein identification minimum of five amino acids per identified peptide and at least one peptide per protein group were required. The false discovery rate was set to 1% at both peptide and protein levels. “Re-quantify” was enabled, and “keep low scoring versions of identified peptides” was disabled. Statistical analysis was performed with Perseus bioinformatics platform which is part of MaxQuant (COX AND MANN 2008).

**qRT-PCR**

Total RNA was extracted using Trizol (Ambion) followed by isolation using Direct-Zol RNA Miniprep (Zymo Research) following the manufacturers’ protocols.

Relative transcript levels were measured using total RNA extracts from 10 females of control (w1118) and *mir-310s* mutant (KT40/KT40) genotypes kept under well-fed or starved condition for 10 days using 3 biological replicates. To synthesize total cDNA, High-Capacity reverse transcription kit (Applied Biosystems) and random primers were used. Quantitative PCR (qPCR) was performed using SYBR green master mix (Applied Biosystems) using a StepOne Plus thermocycler (Applied Biosystems) according to manufacturer’s instructions. The gene *Rpl32* was used as an endogenous control. Primers for qPCR for each gene were designed using Lasergene software (Table S11). The amplicons were selected to be intron spanning. If that was not possible, additional DNAse (Zymo Research) treatment of the RNA samples was performed and reverse transcriptase negative controls were included.
Relative miRNA levels were measured using RNA extracts from 5 ovaries from 7 day well-fed or starved control (w^{118}/Oregon-R-C) females in at least 3 biological replicates. TaqMan microRNA assays (Applied Biosystems) and High-Capacity reverse transcription kit were used to synthesize cDNA specific to mir-310, mir-312, and 2S rRNA as an endogenous control. qPCR was performed using the Taqman qPCR master mix (Applied Biosystems) using a StepOne Plus thermocycler.

For the relative quantitative analysis, average C_T values of technical replicates were first normalized by subtraction of the housekeeping gene expression (Rpl32 for transcript expression and 2S rRNA for miRNA expression) and then of the gene of interest expression in the well-fed controls. Relative expression levels were obtained with these calculated ΔΔC_T values using the formula 2^{-ΔΔCT}. Statistical analysis was done using non-paired two-tailed Student’s t-test.

**Immunohistochemistry**

Adult ovaries were dissected in cold 1X PBS and fixed for 10-15 minutes in 4% formaldehyde (Polysciences Inc.) at room temperature. The subsequent staining procedure was performed as described (KONIG AND SCHERBATA 2013). The following antibodies were used with the indicated dilutions: mouse monoclonal anti-Adducin (1:50), anti-LaminC (1:20), anti-Fasciclin III (1:50), and anti-β-Gal (1:25), rat monoclonal anti-DE-Cadherin (1:25) (Developmental Studies Hybridoma Bank); chicken polyclonal anti-GFP (1:5000, Abcam); guinea pig polyclonal anti-Hh (1:100, gift from Acaimo González-Reyes); rabbit polyclonal anti-PH3 (1:5000, Upstate Biotechnology); goat secondary antibodies Alexa 568 anti-mouse, Alexa 488 anti-rat, Alexa 488 anti-rabbit, Alexa 488 anti-chicken, and Alexa 568 anti-guinea pig (1:500, Invitrogen). To stain cell nuclei, DAPI dye
(Sigma) was used. All samples were mounted on glass slides in 1X PBS with 70% glycerol and 3% n-propyl gallate. Fluorescence images of the stained tissues were taken with confocal laser-scanning microscope (Zeiss LSM 700) and processed with Adobe Photoshop software.

Luciferase Assay

The reporter constructs with a short 3’UTR fragment of each gene containing the mir-310s binding site was cloned downstream of Renilla luciferase gene (Table S11). The same vector contained an unmodified Firefly luciferase gene, activity of which served as an internal transfection control for each experiment and for the normalization of Renilla luciferase signal. Drosophila S2 cells were kept in Schneider’s Drosophila medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (GE healthcare), 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco). The cells were split 1:6 the day before transfection and seeded into 96 well plates. All wells were transfected with 5ng actin Gal4, 20ng of UAS-mir-310s (gifts from Eric Lai), and 10ng psiCHECK™-2 vectors (Promega) with or without the 3’UTR fragment of the respective gene using Effectene® Transfection Reagent (Qiagen). Experiments were done in triplicates. Firefly and Renilla luciferase activities were measured 72h after transfection using Dual-Glo® Luciferase Assay System (Promega) by Wallac 1420 luminometer (PerkinElmer). For analysis, the Renilla luciferase signal was divided by Firefly luciferase signal to normalize the data to the amount of cells transfected in each well. Next, this ratio was normalized to the control, unmodified Renilla luciferase signals, for each respective miRNA overexpression experiment.

Coupled Colorimetric Assay (CCA)
Total body fat content of the flies was measured by CCA as described (GALIKOVA et al. 2015). Five female flies were homogenized in 1000µl 0.05% TWEEN® 20 (Sigma) and incubated at 70°C for 5 minutes. Samples were cleared by centrifuging at 3000g for 3 minutes and the supernatant was used for subsequent colorimetric analyses. To measure the triglyceride (TAG) equivalent amounts, we used 200µl of prewarmed (37°C) Triglycerides Reagent (Thermo Scientific™) with 50µl of the well-fed and 75µl of the starved samples measuring the absorbance at 540nm after incubation at 37°C for 30 minutes. Absolute TAG equivalent amounts were calculated with help of serial dilutions of Thermo Trace Triglyceride standard (Thermo Scientific™) and calculated standard curve. For normalization, we measured total protein content of the samples using BCA Protein Assay Reagent (Thermo Scientific Pierce), where we used 50µl of the samples with 200µl BCA-mix and measured absorbance at 570nm after an incubation for 30 minutes at 37°C. Absolute protein contents of the samples were calculated with the help of a standard curve obtained using measurements of serial dilutions of bovine serum albumin standard. Both absorbance measurements were done in 96 well microtest plates (Sarstedt) using a Benchmark Microplate Reader (Biorad).

Fat bodies were visualized from non-fixed dorsal carcass preparations using Bodipy493/503 (38 µM; Invitrogen) to label lipid droplets, CellMask™ Deep Red (5 µg/mL; Invitrogen) to label plasma membrane, and DAPI (3,6 µM; Invitrogen) to label nuclei (GALIKOVA et al. 2015).

**Co-immunoprecipitation**

Whole lysates were prepared from approximately 1-week-old male and female flies, which were kept on nutrient rich food for 2-3 days and harvested by snap freezing in liquid nitrogen. Three biological
replicates of 750mg of both control (w^{1118}) and Rab23::YFP::4xmyc flies were homogenized by grinding in 2ml buffer with 20mM Tris (pH 7.4), 150mM NaCl, 5% glycerol, 5mM EDTA, 0.1% Triton™ X-100 (Sigma) and 2X protease inhibitor cocktail (Roche) in a mortar with pestle using liquid nitrogen. Lysates were cleared by three centrifuging steps once for 10 minutes at 15000g and twice at 21000g at 4°C. Next, control and Rab23::YFP::4xmyc lysates were diluted with buffer to 5ml and were added 50µl agarose beads coupled with anti-myc antibodies (Sigma) in 15ml tubes and incubated rotating at 4°C for 100 minutes. To collect the beads, lysates were centrifuged at 100g for 2 minutes at 4°C. The beads were washed 10 times with 700µl buffer at 100g for 30 seconds at 4°C and finally eluted with 50µl warm 2X sample buffer (NuPAGE® LDS Sample Buffer, Novex®). The eluates were analyzed by mass spectrometry with the same workflow used in SILAC analysis described above with the exception for trypsin used for in-gel digestion.

Supplemental References

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