TORC1 regulators Iml1/GATOR1 and GATOR2 control meiotic entry and oocyte development in *Drosophila*

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In single-cell eukaryotes the pathways that monitor nutrient availability are central to initiating the meiotic program and gametogenesis. In *Saccharomyces cerevisiae* an essential step in the transition to the meiotic cycle is the down-regulation of the nutrient-sensitive target of rapamycin complex 1 (TORC1) by the increased minichromosome loss 1/ GTPase-activating proteins toward Rags 1 (Iml1/ GATOR1) complex in response to amino acid starvation. How metabolic inputs influence early meiotic progression and gametogenesis remains poorly understood in metazoans. Here we define opposing functions for the TORC1 regulatory complexes Iml1/ GATOR1 and GATOR2 during *Drosophila* oogenesis. We demonstrate that, as is observed in yeast, the Iml1/GATOR1 complex inhibits TORC1 activity to slow cellular metabolism and drive the mitotic/meiotic transition in developing ovarian cysts. In *iml1* germline depletions, ovarian cysts undergo an extra mitotic division before meiotic entry. The TORC1 inhibitor rapamycin can suppress this extra mitotic division. Thus, high TORC1 activity delays the mitotic/ meiotic transition. Conversely, mutations in Tor, which encodes the catalytic subunit of the TORC1 complex, result in premature meiotic entry. Later in oogenesis, the GATOR2 components Mio and Seh1 are required to oppose Iml1/GATOR1 activity to prevent the constitutive inhibition of TORC1 and a block to oocyte growth and development. To our knowledge, these studies represent the first examination of the regulatory relationship between the Iml1/GATOR1 and GATOR2 complexes within the context of a multicellular organism. Our data imply that the central role of the Iml1/GATOR1 complex in the regulation of TORC1 activity in the early meiotic cycle has been conserved from single cell to multicellular organisms.

meiosis | Iml1 | GATOR1 | GATOR2 | Drosophila

In yeast, the inhibition of the nutrient-sensitive target of rapamycin complex 1 (TORC1) in response to amino acid limitation is essential for cells to transit from the mitotic cycle to the meiotic cycle (1, 2). In response to amino acid starvation, the Iml1 complex, comprising the Iml1, Npr2, and Npr3 proteins in yeast and the respective orthologs DEPDC5, Nprl2, and Nprl3 in mammals, inhibits TORC1 activity (3–5). The Iml1 complex, which has been renamed the "GTPase-activating proteins toward Rags 1" (GATOR1) complex in higher eukaryotes, functions as a GTPase-activating protein complex that inactivates RagsA/B or Gtr1 in mammals and yeast, respectively, thus preventing the activation of TORC1 (3, 4). In the yeast *Saccharomyces cerevisiae*, mutations in the Iml1 complex components Npr2 and Npr3 result in a failure to down-regulate TORC1 activity in response to amino acid starvation and block meiosis and sporulation (6–9). As is observed in yeast, in *Drosophila*, Nprl2 and Nprl3 mediate a critical response to amino acid starvation (10). However, their roles in meiosis and gametogenesis remain unexplored.

Recent reports indicate that the Iml1, Npr2, and Npr3 proteins are components of a large multiprotein complex originally named the "Seh1-associated" (SEA) complex in budding yeast and the "GATOR" complex in higher eukaryotes (3, 11). The SEA/GATOR complex contains eight highly conserved proteins. The three proteins described above, Iml1/DEPDC5, Npr2/Nprl2, and Npr3/Nprl3, form the Iml1/GATOR1 complex and inhibit TORC1 (3, 4). The five remaining proteins in the complex, Seh1, Sec13, Sea4/Mio, Sea2/WDR24, and Sea3/WDR59, which have been designated the "GATOR2" complex in multicellular organisms, oppose the activity of Iml1/GATOR1 and thus promote TORC1 activity (3, 4, 12).

Little is known about the physiological and/or developmental requirements for the GATOR2 complex in multicellular organisms. However, in *Drosophila* the GATOR2 components Mio and Seh1 interact physically and genetically and exhibit strikingly similar ovarian phenotypes, with null mutations in both genes resulting in female sterility (13, 14). In *Drosophila* females, oocyte development takes place within the context of an interconnected germline syncytium, also referred to as an "ovarian cyst" (15). Ovarian cyst formation begins at the tip of the germarium when a cystoblast, the daughter of a germline stem cell, undergoes four synchronous divisions with incomplete cytokinesis to produce 16 interconnected cells. Actin-stabilized cleavage furrows, called "ring canals," connect cells within the cyst (16). Each 16-cell cyst develops with a single oocyte and 15 polyploid nurse cells which ultimately are encapsulated by a somatically derived layer of follicle cells to produce an egg chamber (17).

Significance

The target of rapamycin complex 1 (TORC1) promotes cell growth and anabolic metabolism. In yeast, entry into meiosis is contingent on the down-regulation of TORC1 activity by the increased minichromosome loss 1/GTPase-activating proteins toward Rags 1 (Iml1/GATOR1) complex in response to amino acid starvation. Here we define the developmental requirements for the TORC1 regulators Iml1/GATOR1 and GATOR2 during *Drosophila* oogenesis. We demonstrate that, as is observed in yeast, the Iml1/GATOR1 complex down-regulates TORC1 activity to facilitate the mitotic/meiotic transition in *Drosophila* ovarian cysts. Later in oogenesis, components of the GATOR2 complex oppose the activity of GATOR1 to enable a rise in TORC1 activity that drives oocyte development and growth. Thus, a conserved nutrient stress pathway has been incorporated into a developmental program that regulates meiotic progression in *Drosophila*.

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Each ovary is comprised of ~15 ovarioles that consist of a single germarium followed by a string of egg chambers in successively older stages of development. In *mio-* and *seh1*-mutant egg chambers, the oocyte enters the meiotic cycle, but as oogenesis proceeds, the oocyte fate and the meiotic cycle are not maintained stably (13, 14). Ultimately, a large fraction of *mio* and *seh1* oocytes enter the endocycle and develop as polyploid nurse cells. A mechanistic understanding of how *mio* and *seh1* influence meiotic progression and oocyte fate has remained elusive.

Here we demonstrate that the Iml1/GATOR1 complex downregulates TORC1 activity to promote the mitotic/meiotic transition in *Drosophila* ovarian cysts. We find that depleting *iml1* in the female germ line delays the mitotic/meiotic transition, so that ovarian cysts undergo an extra mitotic division. Conversely, mutations in *Tor* result in premature meiotic entry before the completion of the four mitotic divisions. Moreover, we demonstrate that in the female germ line, the GATOR2 components Mio and Seh1 are required to oppose the TORC1 inhibitory activity of the Iml1/GATOR1 complex to prevent the constitutive downregulation of TORC1 activity in later stages of oogenesis. To our knowledge, these studies represent the first examination of the regulatory relationship between Iml1/GATOR1 and GATOR2 components within the context of a multicellular animal. Finally, our data reveal a surprising tissue-specific requirement for the GATOR2 complex in multicellular organisms and suggest a conserved role for the SEA/GATOR complex in the regulation of TORC1 activity during gametogenesis.

Results

The TORC1 Inhibitor Iml1 Regulates the Mitotic/Meiotic Switch During *Drosophila* Oogenesis. The genome of *Drosophila melanogaster* contains a single IML1 homolog encoded by the gene *CG12090* and shares 27% identity with and 49% similarity to yeast IML1 and 36% identity with and 51% similarity to the human IML1 homolog DEPDC5. In the work presented here, *Drosophila CG12090* is referred to as "*iml1*." To examine the role of the Iml1/GATOR1 complex in the regulation of the early meiotic cycle, we generated transgenic lines that express shRNA which target the *iml1* transcript under the control of the GAL4-upstream activating site (UAS) (18). These transgenic lines were used for the tissue-specific depletion of *iml1,* the catalytic subunit of the Iml1/GATOR1 complex (4). When the *iml1* RNAi constructs were expressed in the female germ line using the *MTD-GAL4* germ line-specific driver, a large fraction of ovarian cysts delayed meiotic commitment and underwent a fifth mitotic division to produce 32-cell cysts with a single oocyte as visualized by the oocyte-specific marker Orb (Fig. 1 *A* and *B*) (19, 20). Oocytes in egg chambers that contain 32 cells have five ring canals, consistent with the ovarian cysts having undergone an extra mitotic division (Fig. 1 *C* and *D*). When two independent *iml1 RNAi* lines were coexpressed in the female germ line, the percentage of ovarian cysts that underwent an extra mitotic division before meiotic commitment increased significantly (Fig. 1*E*). The additive nature of the two independent *iml1 RNAi* lines, which target different regions of the *iml1* transcript, demonstrates that the phenotype is not caused by off-target effects. Moreover, we found that codepleting a second Iml1/GATOR1 component, *nprl2*, dramatically enhanced the penetrance of the *iml1 RNAi* 32 cell cyst phenotype (Fig. 1*E*). Thus, depleting components of the Iml1/GATOR1 complex in the female germ line results in a delayed and/or aberrant mitotic/meiotic transition in *Drosophila* ovarian cysts.

We reasoned that the delayed entry into the meiotic cycle in the *iml1 RNAi* depletions was caused by inappropriately high TORC1 activity. To test this model, we examined whether the drug rapamycin, a specific inhibitor of TORC1, could suppress the extra mitotic division observed in *iml1 RNAi* knockdowns. As predicted, we found that feeding *iml1 RNAi*-depletion females rapamycin dramatically decreased the number of ovarian cysts

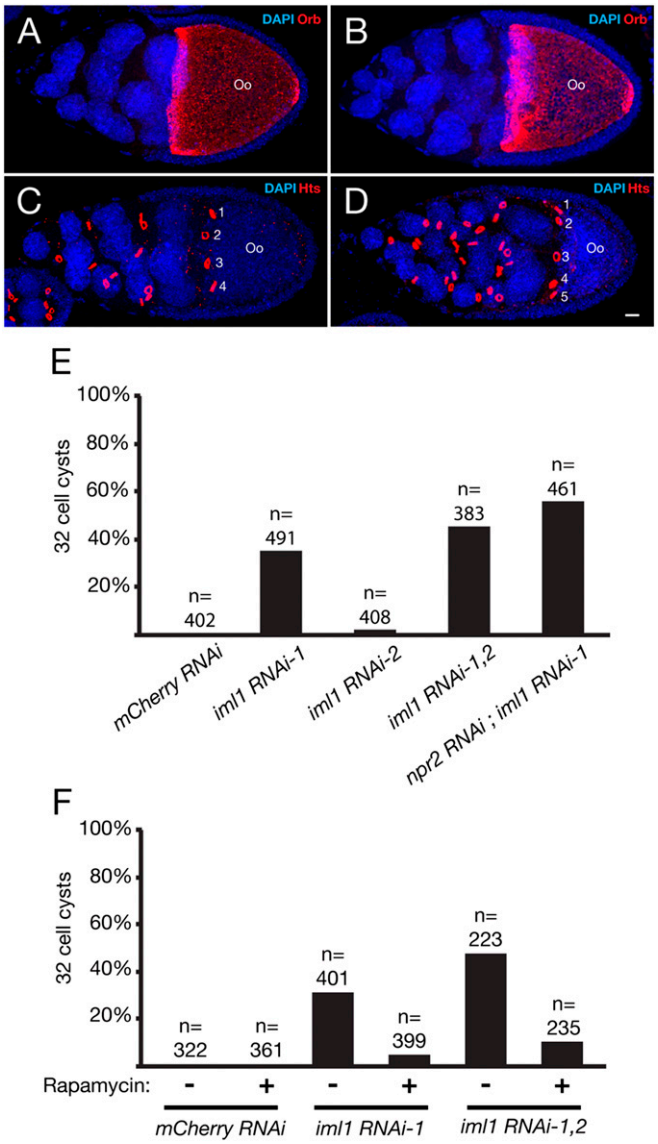

Fig. 1. *iml1* depletions delay mitotic exit in *Drosophila* ovarian cysts. (*A* and *B*) Ovaries are stained with DAPI (DNA; blue) and anti-Orb (oocyte marker; red). (*A*) MTD-Gal4>*mCherry RNAi*. (*B*) MTD-Gal4>*iml1 RNAi*. (*C* and *D*) Ovaries are stained with DAPI (DNA; blue) and anti-Hts (ring canal marker; red). Numbers denote oocyte ring canals. (*C*) MTD-Gal4>*mCherry RNAi*. (*D*) MTD-Gal4>*iml1 RNA*. (Scale bar: 10 μm.) (*E*) Percentage of egg chambers that contain 32-cell cysts. (*F*) Rescue of 32-cell cyst phenotype by rapamycin.

that delayed meiotic commitment and underwent a fifth mitotic division (Fig. 1*F*). Thus, the high TORC1 activity associated with *iml1* depletions influences the timing of mitotic/meiotic transition. Taken together, our data indicate that, as is observed in yeast, during *Drosophila* oogenesis the Iml1/GATOR1 complex promotes a timely entry into the meiotic cycle through the inhibition of TORC1 activity.

We find that high TORC1 activity during early ovarian cyst development delays the mitotic/meiotic transition during *Drosophila* oogenesis. To determine if low TORC1 activity has the opposite effect on the timing of the mitotic/meiotic transition, we generated homozygous germline clones of a *Tor*-null allele, $Tor^{\Delta P}$. During the development of a wild-type egg, ovarian cysts undergo four mitotic divisions to produce a 16-cell cyst with an invariant pattern of interconnections. Subsequently, the two pro-oocytes progress to pachytene and form mature synaptonemal complexes.

Previous work has determined that Tor activity controls germline cyst proliferation and survival (21, 22). Indeed, germline clones of strong *Tor* alleles undergo apoptosis at the 8- or 16-cell stage and thus never bud off from the germarium to generate a mature egg chamber (21, 22). We were interested in determining whether germline clones of a *Tor*-null allele enter meiosis prematurely before undergoing apoptosis. Intriguingly, we found that in $Tor^{\Delta P}$ germline clones, 67% ($n = 9$) of eight-cell cysts contain pro-oocytes that have progressed to pachytene (Fig. 2 and Fig. S1). In contrast, as has been reported by others (23–27), we never observed a pachytene configuration in wild-type eight-cell cysts ($n = 10$). Thus, low $Tor^{\Delta P}$ activity results in the premature transition of ovarian cysts from the mitotic cycle to the meiotic cycle.

The GATOR2 Components Mio and Seh1 Promote TORC1 Activation in the Female Germ Line. Our data support the model that the Iml1/GATOR1 complex down-regulates TORC1 activity to slow cellular metabolism and promote meiotic entry in dividing ovarian cysts. However, high TORC1 activity is required to drive oocyte growth and development in later stages of oogenesis (21, 22, 28). Thus, we reasoned that the down-regulation of TORC1 activity by the Iml1/GATOR1 complex in early ovarian cysts must be transient. Recent evidence indicates that the TORC1 inhibitory activity of the Iml1/GATOR1 complex is restrained by a second multiprotein complex, GATOR2 (3, 4). In *Drosophila* the GATOR2 components Mio and Seh1 are required in the female germ line for meiotic maintenance and oocyte development (13, 14). Our observations on the early role of the Iml1/GATOR1 complex in the development of ovarian cysts raise the intriguing possibility that the ovarian defects observed in *mio* and *seh1* mutants result from the constitutive inhibition of TORC1 by the GATOR1 complex in the absence of a functional GATOR2 complex.

To test this model, we examined the role of the GATOR2 component *mio* in the regulation of TORC1 activity and growth in germline and somatic cells by generating homozygous clones of a *mio*-null allele, mio^2. Scoring homozygous mio^2 clones allowed the detection of subtle differences in the growth rates of homozygous mio^2-mutant and adjacent $mio^{2/+}$ heterozygous wild-type cells. We determined that egg chambers that contained germline clones of mio^2 were dramatically smaller and had nurse cells with decreased ploidy values (DNA content) relative to adjacent wild-type $mio^{2/+}$ egg chambers (Fig. 3*A*). Importantly, the block to egg chamber growth in mio^2 germline clones is not dependent on the lack of an oocyte. Egg chambers that contain mio^2 germline clones that maintain an oocyte still exhibit dramatically reduced growth relative to similarly staged wild-type egg chambers (Fig. 3*A*). In contrast to observations in the germ line, clones of mio^2 in somatic cell types, including the fat body and the somatic follicle cells of the ovary, resulted in only a small decrease, if any, in cell ploidy or cell size (Fig. 3 *B* and *C*). Taken together, our data demonstrate that there is a tissue-specific requirement for the GATOR2 component Mio for cell growth in the female germ line.

To determine if the blocking of growth in *mio*-mutant ovaries correlates with a decrease in TORC1 activity, we compared the phosphorylation status of S6 kinase, a downstream TORC1 target, in ovaries from wild-type, *mio*, and *seh1* mutant females (29). Mio and Seh1 are both components of the GATOR2 complex and are predicted to form a heterodimer (30). We compared TORC1 activation in both mutant and wild-type ovaries with that observed in whole males, which are comprised primarily of somatic tissues. Consistent with the retarded growth observed in egg chambers containing *mio* germline clones, we observed an approximately fourfold decrease in TORC1 activity in *mio* and *seh1* mutant ovaries relative to ovaries from wild-type females (Fig. 3*D*). A comparatively small decrease in TORC1 activity was observed in *mio* mutant males, but no decrease in TORC1 activity was observed in *seh1* mutant males (Fig. 3*E*). These data suggest that *mio* and *seh1* are required for the full activation of TORC1 in the female germ line but play a relatively minor role in the activation of TORC1 in many somatic cell types. Thus, the requirement for the GATOR2 complex for the activation of TORC1 is tissue specific.

The inhibition of TORC1 activates catabolic metabolism and autophagy (31). We reasoned that, if the *mio* and *seh1* ovarian phenotypes are caused by the failure to deactivate the TORC1 inhibitor Iml1/GATOR1 after meiotic entry, then mutant ovarian cysts should continue to undergo catabolic metabolism throughout oogenesis. To assess the metabolic state of *mio* and *seh1* mutant ovaries, we stained ovaries with LysoTracker (Life Technologies), a marker for acidic endomembrane compartments that highlights lysosomes and autolysosomes. Consistent with the constitutive activation of catabolic metabolism, we found that, even when mutant females have access to a protein source, *mio* and *seh1* mutant egg chambers are filled with large LysoTracker-positive puncta (Fig. 4 *A–D*). We determined that these structures are autolysosomes using two criteria. First, these large puncta were positive for Atg8a, known as "LC3" in mammals, a protein that targets autophagosomes and autolysosomes (Fig. 4 *F* and *G*) (32). Second, transmission electron microscopy revealed that *mio* and *seh1* mutant ovaries are filled with very

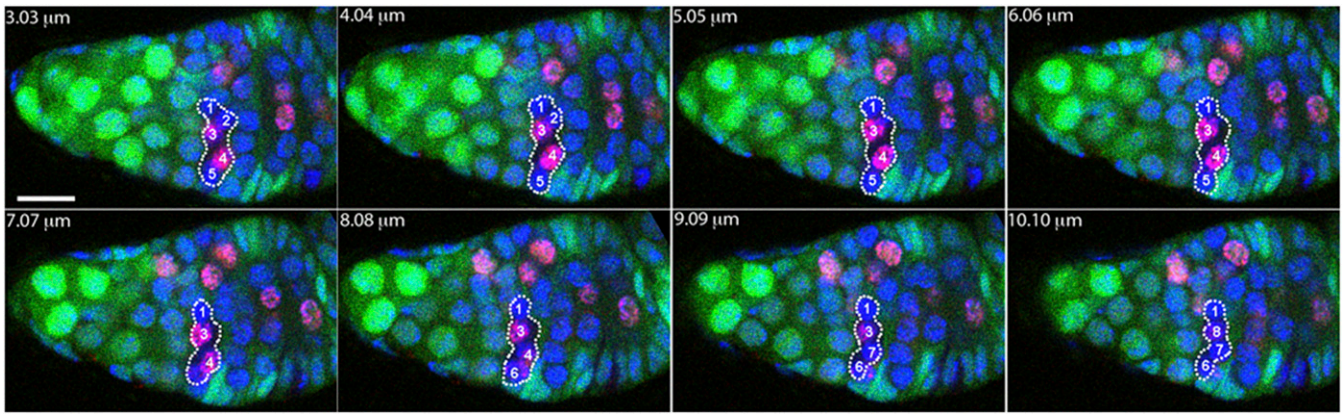

Fig. 2. $Tor^{\Delta P}$ germline clones enter meiosis prematurely. A $Tor^{\Delta P}$ eight-cell germline clone (denoted by the dotted line) contains pro-oocytes that have progressed to pachytene. The mutant germline clone is marked by the absence of GFP (green). The synaptonemal complex is highlighted by anti-C(3)G (red). Individual nuclei are marked by DAPI. The separated channels for each marker are presented separately in Fig. S1. Numbers shown within the dotted lines represent individual nuclei.

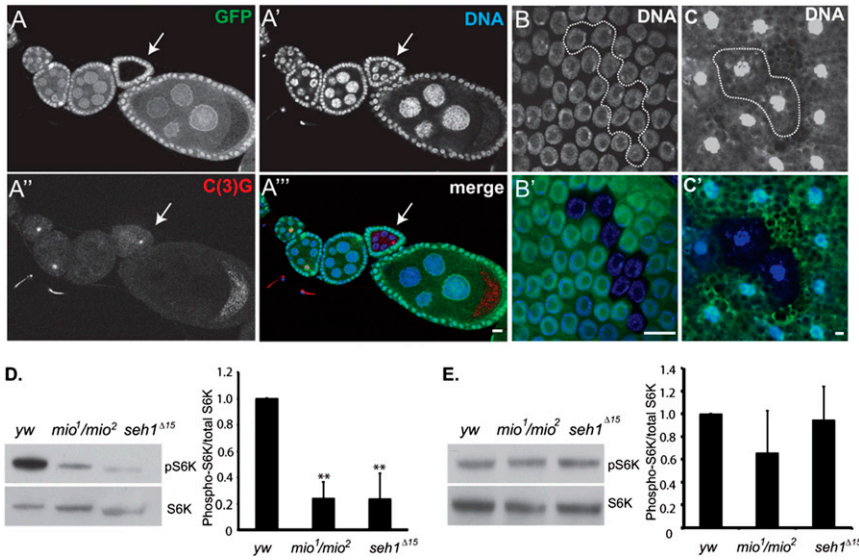

Fig. 3. Mio and Seh1 promote TORC1 activation in the female germ line. (*A*–*A*′′′) Ovariole containing a *mio*^{2} germline clone stained with anti-GFP (green) (*A*), DAPI (blue) (*A*′), and anti-C(3)G (red) (*A*′′). The mutant egg chamber is marked by the absence of GFP (arrow). Note that the egg chamber containing the *mio*^{2} germline clone contains an oocyte, as indicated by the C(3)G staining. (*B*) *mio*^{2}-mutant follicle cell clones in a stage-10 egg chamber stained with DAPI (blue) and anti-GFP (green). (*C*) *mio*^{2}-mutant fat body clone stained with Hoechst (blue). For *B* and *C*, *mio*^{2} homozygous clones were identified by the absence of GFP. (Scale bars: 10 μm). (*D* and *E*) Protein isolated from female ovaries (*D*) and whole males (*E*) was analyzed by Western blot. Quantification of phospho-S6K levels relative to the total S6K is shown. Error bars represent SD from four independent experiments. $^{**}P < 0.01$. In *D* and *E*, *yw* stands for *yellow, white* and serves as a wild-type control.

large single-membrane vesicles that contained partially digested material (Fig. 4 *H* and *I*). Thus, *mio* and *seh1* ovarian cysts accumulate autolysosomes throughout oogenesis consistent with the constitutive inhibition of TORC1 activity in the female germ line. Importantly, the activation of catabolic metabolism is not caused by the lack of an oocyte. *egl* mutants, which also produce egg

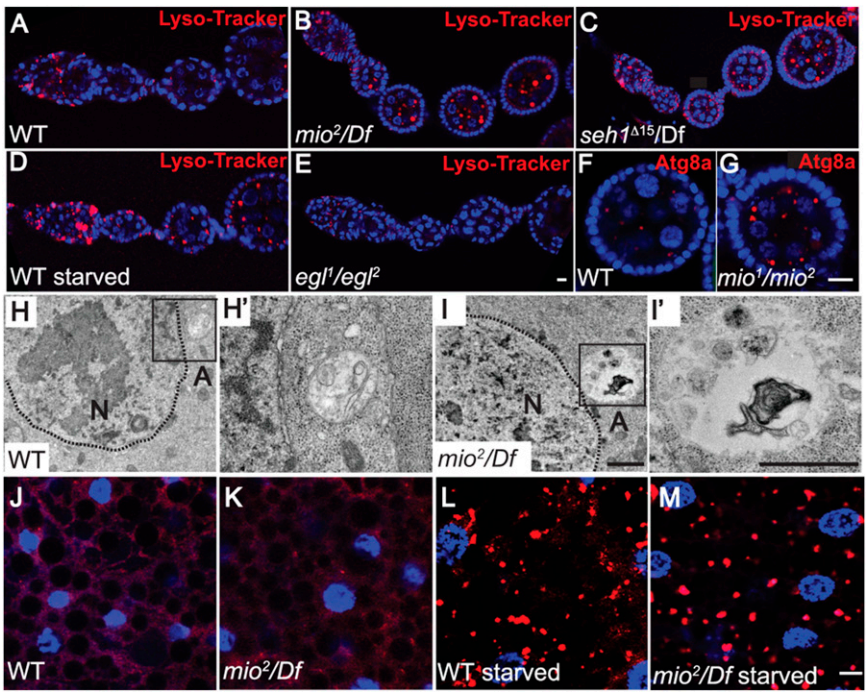

Fig. 4. *mio*- and *seh1*-mutant egg chambers accumulate autolysosomes. (*A*–*E*) Ovarioles from wild-type fed (*A*), *mio*^{2}/*Df* fed (*B*), *seh1*$^{\Delta 15}$ fed (*C*), wild-type starved (*D*), and *egl*^{1}/*egl*^{2} fed (*E*) females stained with LysoTracker and Hoechst. (Scale bar: 10 μm.) (*F* and *G*) Live-cell imagining of mCherry-Atg8a in wild-type (*F*) and *mio*^{1}/*mio*^{2}-mutant (*G*) egg chambers. (Scale bars: 10 μm.) (*H* and *I*) Transmission electron microscope images of autolysosomes from wild-type starved (*H*) and *mio*^{2}/*Df* fed (*I*) egg chambers. Autolysosomes are shown at higher magnification for both wild-type (*H*′) and *mio*-mutant (*I*′) egg chambers. A, autolysosomes; N, nucleus. (Scale bars: 1 μm.) (*J*–*L*) Fat bodies from wild-type fed (*J*), *mio*^{2}/*Df* fed (*K*), wild-type starved (*L*), and *mio*^{2}/*Df* starved (*M*) third-instar larvae were stained with LysoTracker and Hoechst. (Scale bar: 10 μm.)

chambers with 16 nurse cells because of a defect in ovarian cyst polarity, do not have increased LysoTracker staining (Fig. 4*E*) (33). Thus, the increased autophagy observed in *mio* and *seh1* mutants is not triggered by the absence of an oocyte but likely reflects a direct role for *mio* and *seh1* in metabolic regulation.

To determine if the *mio* and *seh1* mutants activate the autophagy pathway in somatic tissues, we stained fat bodies from wild-type, *mio*, and *seh1* mutant third-instar larvae with LysoTracker. As predicted by the limited effects on growth and TORC1 activity previously observed in *mio* and *seh1* mutant somatic tissues, the fat bodies from well-fed *mio* and *seh1* mutant third-instar larvae do not accumulate autolysosomes (Fig. 4 *J*–*M* and Figs. S2 and S3). Thus, Mio and Seh1 are specifically required to inhibit catabolic metabolism and the activation of autophagy in the female germ line but are not generally required in somatic tissues under nutrient replete conditions.

The GATOR2 Components Mio and Seh1 Localize to Lysosomes and Autolysosomes. Next, we wanted to determine the intracellular localization of the GATOR2 components Mio and Seh1. Notably, GATOR2 is reported to act as an upstream regulator of Iml1/GATOR1 (3, 12). The Iml1/GATOR1 complex localizes to lysosomes, the site of TORC1 activation, and to autolysosomes (3, 10). We found that Mio-GFP, expressed under its native promoter, as well as GFP-Seh1 expressed in the female germ line using the *MTD-Gal4* driver, localized to LysoTracker-positive structures in ovaries from both fed and starved females (Fig. 5 *A*–*D*). The LysoTracker-positive structures in egg chambers from fed females were considerably fewer in number and smaller than those found in the ovaries from females that had been starved for amino acids. The increased number and size of the puncta found in ovaries from starved females likely is the result of the onset of autophagy. Consistent with this idea, Mio-GFP and GFP-Seh1 colocalize strongly with Atg8a-mCherry, a component of autophagosomes and autolysosomes, under starvation conditions (Fig. S4). Thus, as is observed with the intracellular localization of Iml1/GATOR1, the GATOR2 components Mio and Seh1 target to lysosomes and autolysosomes. Finally, we wanted to determine if Iml1/GATOR1 and GATOR2 components colocalize. To answer this question, we coexpressed GFP-Seh1 and Nprl3-mCherry in the female germ line and found that the proteins colocalize to puncta under both fed and starved conditions (Fig. 5 *E* and *F*). We note, however, that the colocalization of the two tagged proteins within puncta is not always perfectly coincident (Fig. 5 *E″* and *F″*). Thus, Iml1/GATOR1 and GATOR2 components colocalize to lysosomes, which are the site of TORC1 regulation, and autolysosomes.

Mio and Seh1 Prevent the Constitutive Activation of the Gator1 Complex in the Female Germ Line. Our data suggest that the *mio* and *seh1* ovarian phenotypes are caused by the unopposed TORC1 inhibitory activity of the GATOR1 complex in the female germ line. To test this model, we examined the epistatic relationship between *mio* and *seh1* and the GATOR1 components *nprl2* and *nprl3*. In flies with null alleles of both *mio* and *seh1,* egg chambers often develop without an oocyte and cease to grow beyond stage 5 or 6 of oogenesis (Fig. 6 *B* and *G*) (13, 14). We found that depleting *nprl2* and *nprl3* transcripts in the female germ line rescued the 16-nurse-cell and growth phenotypes of *mio*- and *seh1*-null mutants (Fig. 6 *K* and *L*). Furthermore, the *seh1* egg-production defect also is rescued by depleting *nprl2* and *nprl3* (Fig. S5) (14). Thus, in the female germ line, the TORC1 inhibitors *nprl2* and *nplr3* are epistatic to the TORC1 activators *mio* and *seh1.* Finally, we found that *iml1* RNAi codepletion also rescues the ovarian phenotype observed in *seh1* RNAi depletions (Table S1). One possible model to explain the epistasis analysis is that the *mio* and *seh1* ovarian phenotypes are caused by the constitutive inhibition of TORC1 by the Iml1/GATOR1 complex in the female germ line. To test if inappropriate repression of TORC1 is the basis for the *seh1* and *mio* ovarian phenotypes, we used an alternative pathway to up-regulate TORC1 activity in the *mio*- and *seh1*-mutant background. In both *Drosophila* and mammals, the loss of *Tsc1* or *Tsc2* increases the baseline activity of TORC1 (34). Consistent with the *mio* and *seh1* phenotypes resulting from the constitutive inhibition of TORC1, we found that depleting *Tsc1* in the female germ line using RNAi strongly rescues the *mio*- and *seh1*-mutant phenotypes (Fig. 6 *E* and *J*). These experiments confirm that the *mio* and *seh1* ovarian phenotypes, including the inability to maintain the meiotic cycle and the oocyte fate, are caused by low TORC1 activity in the female germ line.

Discussion

Here we define the roles of the TORC1 regulators Iml1/GATOR1 and GATOR2 during *Drosophila* oogenesis. We demonstrate that the Iml1/GATOR1 complex down-regulates TORC1 activity to promote the mitotic/meiotic transition in ovarian cysts, whereas the GATOR2 components Mio and Seh1 are required to oppose the activity of the GATOR1 complex to prevent the constitutive down-regulation of TORC1 activity in later stages of oogenesis (Fig. 7). To our knowledge, our studies represent the first

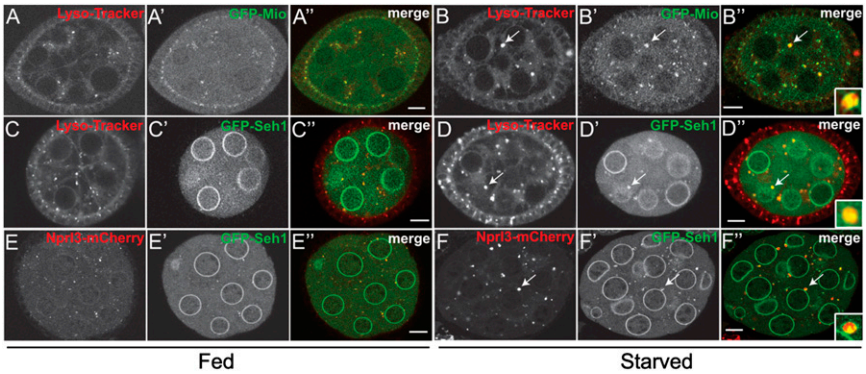

Fig. 5. Mio and Seh1 localize to lysosomes and autolysosomes. Live-cell imaging of *Drosophila* egg chambers from females cultured on fly medium with wet yeast (fed; *A*, *C*, and *E* series) or PBS-agar with 20% sucrose (starved; *B*, *D*, and *F* series). (*A* and *B*) Mio-GFP colocalized with LysoTracker Red ($n > 90$ egg chambers). (*C* and *D* series) GFP-Seh1 colocalized with LysoTracker Red ($n > 18$ egg chambers). (*E* and *F* series) Nprl3-mCherry colocalized with GFP-Seh1 ($n > 20$ egg chambers). MTD-Gal4 driver was used to drive the expression of GFP-Seh1 and Nprl3-mCherry. Mio-GFP was expressed from the endogenous *mio* promoter. (Scale bars: 10 μm.)

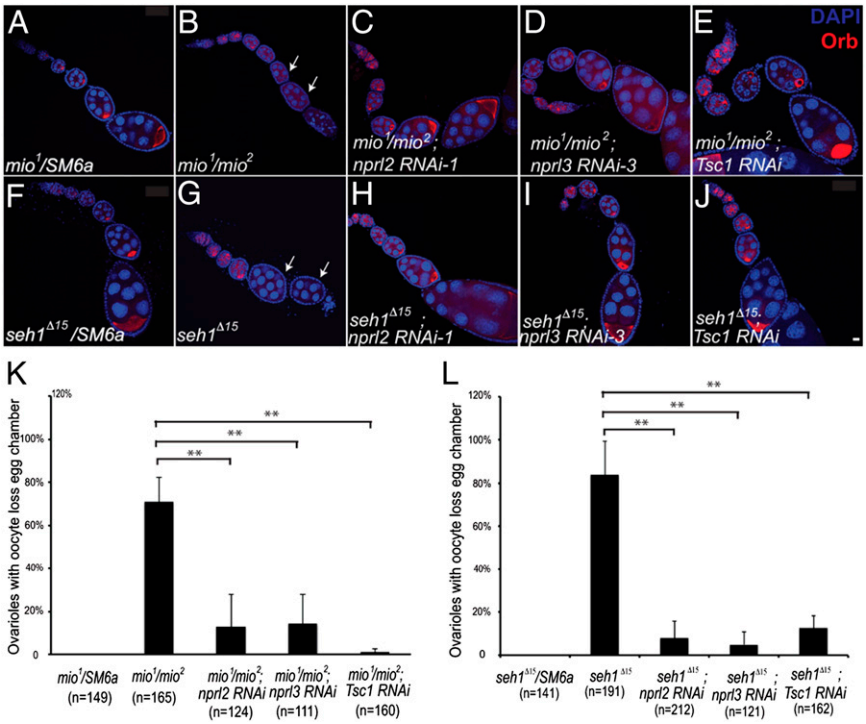

Fig. 6. *nprl2* and *nprl3* are epistatic to *mio* and *seh1* in the *Drosophila* ovary. (*A–J*) Ovaries stained with DAPI (DNA, blue) and anti-Orb (oocyte marker, red): *mio*^{1}*/SM6a; nos-Gal4* (*A*), *mio*^{1}*/mio*^{2}*; nos-Gal4* (*B*), *mio*^{1}*/mio*^{2}*; nos-Gal4/UAS-nprl2 RNAi-1* (*C*), *mio*^{1}*/mio*^{2}*; nos-Gal4/UAS-nprl3 RNAi-3* (*D*), *nos-Gal4; mio*^{1}*/mio*^{2}*; UAS-Tsc1 RNAi* (*E*), *seh1*$^{\Delta 15}$*/SM6a; nos-Gal4* (*F*), *seh1*$^{\Delta 15}$*; nos-Gal4* (*G*), *seh1*$^{\Delta 15}$*; nos-Gal4/UAS-nprl2 RNAi-1* (*H*), *seh1*$^{\Delta 15}$*; nos-Gal4/UAS-nprl3 RNAi-3* (*I*), and *seh1*$^{\Delta 15}$*; nos-Gal4/UAS-Tsc1 RNAi* (*J*). Note that the egg chambers from *mio*^{1}*/mio*^{2} (*B*) and *seh1*$^{\Delta 15}$ (*G*) females often have no oocyte beyond stage 5. Arrows in *B* and *G* indicate egg chambers with no oocyte. (Scale bar: 10 μm.) (*K* and *L*) Percentage of ovarioles in *mio*- (*K*) or *seh1*- (*L*) mutant background that contain at least one egg chamber with no oocyte. *n*, number of ovarioles analyzed. Error bars represent SD from three independent experiments. $^{**}P < 0.01$.

examination of the regulatory relationship between the Iml1/GATOR1 and GATOR2 complexes within the context of a multicellular organism and reveal surprising tissue-specific functions for these conserved TORC1 regulators.

In yeast, meiotic entry requires the down-regulation of TORC1 by the Iml1/GATOR1 complex in response to amino acid starvation (8, 9). Previous work demonstrated that in *Drosophila* the Iml1/GATOR1 complex mediates an adaptive response to amino acid starvation (10). Here we test the hypothesis that the Iml1/GATOR1 complex also has retained a role in the regulation of the early events of gametogenesis. Consistent with this model, we found that in germline knockdowns of *iml1*, ovarian cysts delay meiotic entry and undergo a fifth mitotic division. This meiotic delay can be suppressed with the TORC1 inhibitor rapamycin. Thus, during *Drosophila* oogenesis the Iml1/GATOR1 complex promotes the transition from the mitotic cycle to the meiotic cycle through the down-regulation of the metabolic regulator TORC1. Increasing TORC1 activity by disabling its inhibitor delays meiotic progression, whereas germline clones of a *Tor*-null allele enter meiosis prematurely. Taken together, our data indicate that the level of TORC1 activity contributes to the timing of the mitotic/meiotic switch in *Drosophila* females and suggest that low TORC1 activity may be a conserved feature of early meiosis in many eukaryotes.

However, in *Drosophila*, meiotic entry is not contingent on amino acid limitation at the organismal level. Indeed, the energy-intensive process of *Drosophila* oogenesis is curtailed dramatically when females do not have access to a protein source (35). Thus, to promote meiotic entry, *Drosophila* females must activate the Iml1/GATOR1 complex in a tissue-specific manner, using a mechanism that is independent of the overall nutrient status of the animal. We can think of at least two models to explain how *Drosophila* females might activate the Iml1/GATOR1 complex specifically in the germ line. In the first model, ovarian cysts locally experience low levels of amino acids during the mitotic cyst divisions and/or at the point of meiotic entry. These low levels of amino acids could reflect a non–cell-autonomous effect: The somatically derived escort cells that surround dividing ovarian cysts may function to create a low amino acid environment that triggers the activation of the Iml1/GATOR1 complex within developing ovarian cysts. Alternatively, the effect may be cell autonomous: The germ cells within dividing ovarian cysts may have a reduced ability to sense and/or import amino acids. In a second model, a developmental signaling pathway that is completely independent of local or whole-animal amino acid status directly activates the Iml1/GATOR1 complex. The identification of the upstream requirements for Iml1/GATOR1 activation in the female germ line will help distinguish between these two models.

Although low TORC1 activity is required during early ovarian cyst development to promote the mitotic/meiotic switch, the dramatic growth of egg chambers later in oogenesis is a metabolically expensive process that is predicted to require high TORC1 activity (10, 21, 22). Our data indicate that the GATOR2 components Mio and Seh1 function to oppose the TORC1-inhibitory activity of the GATOR1 complex in the female germ line. In *mio* and *seh1* mutants, TORC1 activity is constitutively repressed in the germ line of developing egg chambers, resulting in the activation of catabolic metabolism and the blocking of meiotic progression and oocyte development and growth. Previous data indicate that Mio and Seh1 act very early in oogenesis soon after the formation of the 16-cell cyst (13, 14). The *mio* and *seh1* ovarian phenotypes can be rescued by depleting the GATOR1 components *nprl2*, *nprl3*, or *iml1* in the female germ line or by raising baseline levels of TORC1 activity by disabling an alternative TORC1 inhibitory

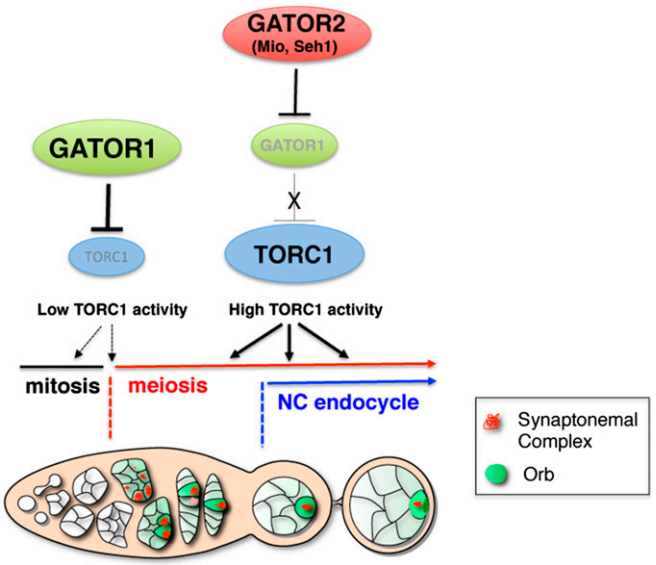

Fig. 7. A model for the role of Iml1/GATOR1 and GATOR2 complexes during *Drosophila* oogenesis. The Iml1/GATOR1 complex down-regulates TORC1 activity to promote the mitotic/meiotic transition after precisely four mitotic cyst divisions. Low TORC1 activity promotes the mitotic/meiotic transition. Whether the inhibition of TORC1 activity by the Iml1/GATOR1 complex is required throughout the ovarian cyst divisions or precisely at the mitotic/meiotic juncture is currently undetermined and thus is represented by dashed arrows. After meiotic entry, the Mio and Seh1 components of the GATOR2 complex oppose the activity of Iml1/GATOR1 complex, thus preventing the constitutive inhibition of TORC1 during later stages of oogenesis. High TORC1 activity is required to drive the nurse cell endoreplication cycle and for the maintenance of the meiotic cycle into later stages of oogenesis.

complex, TSC1/2. These data are consistent with the model that the failure to maintain the meiotic cycle and the oocyte fate in *mio* and *seh1* mutants is a direct result of inappropriately low TORC1 activity in the female germ line brought on by the deregulation of the Iml1/GATOR1 complex.

Notably, null alleles of both *mio* and *seh1* are viable, with many somatic tissues exhibiting no apparent developmental abnormalities and only limited reductions in cell growth (13, 14). Thus, although Mio and Seh1 are critical for the activation of TORC1 and the development of the female gamete, these proteins play a relatively small role in the development and growth of many somatic tissues under nutrient-replete conditions. Whether this small role reflects the fact that components of the Iml1/GATOR1 complex are expressed at low levels in some somatic cell types or that the complex is present but needs to be activated by a signal, such as nutrient stress or a developmental signaling pathway, remains to be elucidated.

In the future it will be important to gain a fuller understanding of the potential environmental and developmental inputs that regulate the activity of the Iml1/GATOR1 and GATOR2 complexes in multicellular organisms. These studies will provide much-needed insight into the basic mechanisms by which both environmental and developmental signaling pathways interface with the metabolic machinery to influence cell growth and differentiation.

Materials and Methods

***Drosophila* Strains and Genetics.** MTD-GAL4 (20), UAS-Tsc1 RNAi, *UAS-mCherrry RNAi*, Df(2L)Exel6007, UASp-GFP-mCherry-Atg8a (36), UASp-mCherry-Atg8a (32), and HS-FLP; Ubi-GFP FRT40A/CyO (37) lines were obtained from the Bloomington Stock Center. HS-FLP; UAS-2×eGFP FRT40A fb-GAL4/CyO and $Tor^{\Delta P}$ were kindly provided by Thomas Neufeld (University of Minnesota) (38, 39). The *UASp-Nprl3-mCherry*, *nprl2 RNAi*, and *nprl3 RNAi* lines were described previously (10). The generation of the *UAS-Tsc1 RNAi* and *UAS-mCherry RNAi* lines were described by Ni et al. (18). All fly stocks were maintained on JAZZ-mix *Drosophila* food (Fisher Scientific) at 25 °C. Rapamycin and amino acid starvation treatments were performed as previously described (10).

Generation of Transgenic Lines. The *seh1*-coding region was amplified and then was inserted into a pENTR-1A vector (Invitrogen). The pENTR-Seh1 plasmids were recombined with pPGW vector (Drosophila Genomics Resource Center) to generate UASp-GFP-Seh1 using the Gateway LR Clonase II enzyme (Invitrogen). The Mio-eGFP transgene contains 978 bp upstream of the *mio* transcription start site, the *mio* 5′ UTR, the *mio*-coding region, the EGFP-coding region, and the *mio* 3′ UTR. These sequences were amplified by PCR and cloned into the pCaSpeR4 vector (40). UASp-GFP-Seh1 and pCaSpeR4-Mio-GFP plasmids were used to generate transgenic lines. All primers used for PCR amplification are listed in Table S2.

To generate the *iml1* RNAi stocks, two shRNA sequences targeting the *iml1* transcript were inserted into Valium 20 and Valium 22 vectors, respectively (18). The plasmids were injected into y^1 v^1; P{y[+t7.7]=CaryP}attP2 and y^1 v^1; P{y[+t7.7]=CaryP}attP40 flies to generate transgenic lines (Genetic Service Inc.). The primers used for shRNA are listed in Table S3.

Western Blot Analysis. *Drosophila* ovaries or whole males were homogenized in RIPA buffer containing Complete Protease Inhibitors and Phosphatase Inhibitors (Roche). Western blots were performed as described previously (10). Antibodies were used at the following concentrations: rabbit anti-P-S6K at 1:500 (Cell Signaling) and guinea pig anti-S6K at 1:10,000 (41). The band intensity was quantified using the Image J analysis tool (National Institutes of Health).

Clonal Analysis. *HS-FLP; Ubi-GFP FRT40A/*mio^2 *FRT40A* females were heat shocked for 60 min in a 37 °C water bath two times/d for 7 d to generate mio^2 homozygous clones in germline and follicle cells before the ovaries were dissected for staining. To generate mio^2 homozygous clones in the fat body, HS-FLP/FRT–mediated recombination was induced in 0- to 8-h embryos through a 90-min, 37 °C heat shock as described previously (38). Fat bodies from third-instar larvae were dissected and stained with Hoechst. To generate $Tor^{\Delta P}$ homozygous clones in the germ line, *HS-FLP; Ubi-GFP FRT40A/*$Tor^{\Delta P}$ *FRT40A* females were heat shocked twice for 1.5 h in a 37 °C water bath two times per day for 2 d. Flies then were fed for 7 d before dissection. Homozygous mio^2 and $Tor^{\Delta P}$ clones were marked by the absence of GFP expression.

Immunofluorescence and Live-Cell Imaging. Immunofluorescence was performed as described (42), using the following antibodies: mouse anti-Orb (1:50; Developmental Studies Hybridoma Bank) (43), rabbit anti-GFP (1:1,000; Invitrogen), mouse anti-Hts (1:50; Developmental Studies Hybridoma Bank) (43), mouse anti-1B1 (1:30; Developmental Studies Hybridoma Bank), and rabbit anti-C3G (1:2,000) (42). Anti-rabbit and anti-mouse Alexa Fluor secondary antibodies (Invitrogen) were used at 1:1,000. Nuclei were visualized by staining the DNA with DAPI (Invitrogen). Images were acquired using an Olympus FV1000 or Leica TCS SP5 confocal microscope. Live-cell images were obtained as previously described (10).

Transmission Electron Microscopy. Ovaries from *mio* mutant or wild-type females were fixed for 1.5 h in 2% (wt/vol) formaldehyde and 2% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2), postfixed in 1% aqueous OsO_4, and stained *en bloc* with 2% (wt/vol) uranyl acetate. Single ovarioles were separated, dehydrated with series of ethanol concentrations, and penetrated with EMbed 812 (Electron Microscopy Sciences).

After treatment, ovarioles were placed on a glass coverslip and embedded in the same resin that subsequently was polymerized at 65 °C for 60 h. The ovarioles were remounted on holders and cut parallel to the glass surface. Thin (70-nm) sections of the egg chambers were cut using a Leica EM UC7 microtome and subsequently were stained with uranyl acetate and lead citrate. The samples were examined on an FEI Tecnai 20 transmission electron microscope operated at 80 kV, and images were recorded on a Gatan Ultrascan CCD camera.

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