A dual role of lola in Drosophila ovary development: regulating stem cell niche establishment and repressing apoptosis

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In Drosophila ovary, niche is composed of somatic cells, including terminal filament cells (TFCs), cap cells (CCs) and escort cells (ECs), which provide extrinsic signals to maintain stem cell renewal or initiate cell differentiation. Niche establishment begins in larval stages when terminal filaments (TFs) are formed, but the underlying mechanism for the development of TFs remains largely unknown. Here we report that transcription factor longitudinals lacking (Lola) is essential for ovary morphogenesis. We showed that Lola protein was expressed abundantly in TFCs and CCs, although also in other cells, and lola was required for the establishment of niche during larval stage. Importantly, we found that knockdown expression of lola induced apoptosis in adult ovary, and that lola affected adult ovary morphogenesis by suppressing expression of Regulator of cullins 1b (Roc1b), an apoptosis-related gene that regulates caspase activation during spermatogenesis. These findings significantly expand our understanding of the mechanisms controlling niche establishment and adult oogenesis in Drosophila.

INTRODUCTION

Longitudinals lacking (lola) is a complex gene in Drosophila melanogaster, encodes at least 20 protein isoforms (Lola A – Lola T) [1–3]. All the Lola isoforms share an N-terminal Broad-Complex, Tramtrack and Bric-à-brac (BTB) domain that is implicated in protein–protein interaction, and 17 isoforms contain unique C-terminal zinc finger (ZF) motifs involved in binding with specific DNA [4–6]. Lola has been shown to play a role in regulating adult midgut homeostasis [7], axon growth and guidance [3, 8, 9], male germline and neuron stem cell maintenance and differentiation [10, 11], embryonic gonad formation and programmed cell death during oogenesis [12, 13]. According to the modENCODE Tissue Expression Data, lola is abundantly expressed in Drosophila ovary (http://flybase.bio.indiana.edu). However, its role in ovarian development has received little attention.

The Drosophila ovary is a powerful model for studying genetics and mechanisms that program maintenance of stem cell niche and development of adult ovary. Drosophila female has a pair of ovaries. Each ovary is composed of 16–20 ovarioles, and an ovariole contains several egg chambers in different developmental stages [14]. Germline stem cells (GSCs) have resided in the tip of gerarium, a structure situated at the apical end of an ovariole. GSCs and their niches constitute functional units to produce eggs to maintain female reproductive capacity. Ovarian GSC niche is composed of several types of somatic cells: terminal filament cells (TFCs), cap cells (CCs) and anterior escort cells (ECs) (Fig. 1A) [15–17]. These cells provide extrinsic signals to GSCs to maintain stem cell identity [18–20]. Terminal filament (TF) formation occurs during the larval stage, and is the beginning point of GSC niche establishment [21]. By the late third instar larval (LL3) and white pre-pupal (WPP) stages, TFCs complete flattening, sorting, intercalation and stacking, and form well-organized TF stacks (Fig. 1A) [22, 23]. The number of GSC niches or ovarioles in adult flies is equal to the number of TFs that form in the larval ovary [24]. At present, only a few genes, including bric-à-brac (bab1/bab2), Lmx1a and engrailed/invected (en/inv), have been reported to be expressed in TFCs and CCs and are involved in the formation of TFs [18, 25, 26]. The genetic events coordinating TF formation and function are still largely unknown.

In adults, oogenesis process is divided into 14 stages according to specific morphological characteristics [14]. It has been reported that programmed cell death (PCD) occurs during early, middle and late stages of oogenesis [27, 28]. In response to starvation, germline cyst cells may undergo PCD within the gerarium, or egg chambers may be degenerated at stage eight [27]. In the late stage of oogenesis, nurse cells ‘dump’ their cytoplasmic contents into the oocyte and undergo PCD [28].

In the present study, we determined the localization of Lola protein in TFCs and CCs of the ovary. We showed that knockdown expression (RNAi) of lola impaired female fertility and ovary morphogenesis. Most ovaries from the lola RNAi lines were significantly smaller in size and did not contain mature eggs. All the abnormal smaller ovaries had fewer ovarioles or lacked distinguishable ovarioles because RNAi of lola impeded TFs formation in the larval stage. RNAi of lola also led to ovarian
apoptosis in adults, which was not starvation-induced PCD nor late stage nurse cell death. Moreover, we showed that lola plays an essential role in Drosophila ovary development by suppressing expression of an apoptosis-related gene, Regulator of sing expression of an apoptosis-related gene, Drosophila an essential role in late stage nurse cell death. Moreover, we showed that apoptosis in adults, which was not starvation-induced PCD nor

RESULTS

Lola protein is expressed in terminal filament cells and cap cells of WPP and adult ovaries

Specific localization of Lola protein in ovary has not been previously reported, although Western blot analysis showed that Lola was present in the larval ovary [12]. Using a Lola antibody to the common BTB domain, we showed that Lola protein was present in larval ovary and adult ovarian gerarium (Fig. 1B, C). In both the larval and adult ovaries, Lola protein was abundant in the terminal filament cells (TFCs) and cap cells (CCs) that constitute the GSC niche, and it was also detected in other cells (Fig. 1B (b‘), C (c)), implying that lola may play a role in the formation of GSC niche.

Lola is required for female fertility

Given that Lola protein is abundant in somatic cells such as TFCs and CCs, we used a somatic driver c587-Gal4 line mated with a lola RNAi line (VDRC 12574) to express inducible lola RNAi in somatic cells. qRT-PCR analysis and immunostaining assay revealed that expression of lola at both the transcriptional and protein levels was significantly decreased in the ovary of c587 > lola12574 than in the control c587 > w1118 (Fig. 2A, B). Importantly, knockdown expression of lola driven by c587-Gal4 in the ovary severely impaired female fecundity (Fig. 2C). To validate c587 > lola RNAi results, we used another lola RNAi line NIG 12052R-1. Since c587 > lola12052R-1 RNAi was lethal in the pupal stage (data not shown), we then used another somatic driver Tj-Gal4 to mate with both lola12574 and lola12052R-1 RNAi lines. Both c587-Gal4 and Tj-Gal4 drivers are expressed in same somatic cells named intermingled cells (ICs) in the larval ovary, which give rise to adult escort cells (ECs) [29, 30]. qRT-PCR results showed that expression of lola transcripts was significantly reduced in the ovary of Tj > lola12574 RNAi line than in the Tj > w1118 control, and the transcriptional level of lola transcripts in the ovary of Tj > lola12052R-1 RNAi line was also reduced compared to the Tj > w1118 line (Fig. 2A). Knockdown expression of lola transcripts driven by both c587-Gal4 and Tj-Gal4 in the ovary had similar impacts on female fecundity (Fig. 2C). These results indicated that lola is required for female fertility. Since both lola12574 and lola12052R-1 RNAi lines had similar effects on female fertility, we used lola12574 RNAi line in most of our following experiments.

Lola is required for ovariode morphogenesis

To explore the underlying mechanism that lola affects female fertility, ovaries from 3-day-old c587 > lola12574, Tj > lola12574 and Tj > lola12052R-1 RNAi lines as well as c587 > w1118 and Tj > w1118 control flies were dissected and stained to examine ovarian development. We found that in the c587 > lola12574 RNAi females, 48.3% of the ovaries had one ovary with significantly smaller size (Fig. 3A (b, c)), and 27.6% of the ovaries had a pair of smaller ovaries...
were observed in the smaller ovaries (Fig. 3A (b, c)). In the ovary of the control c587 > W^{118} line (Fig. 4A (a)), a result consistent with that of the adult ovaries (Fig. 4B). Ovaries from the c587 > W^{118} RNAi line (green brackets in b and b'), female fertility tests were performed in the above fly lines. Significant difference was determined by one-way ANOVA followed by a post hoc Tukey's HSD test and indicated by asterisks: **** p < 0.0001; ns, non-significant. Scale bar: 10 µm in B.

**Lola is required for GSC niche establishment**

Since the number of ovarioles in adult flies matches the number of TFs formed in the larval ovary [24], we speculate that knockdown of *lola* expression may affect the formation of TF in the larval ovary. To investigate this possibility, ovaries from the c587 > lola^{12574} RNAi and c587 > W^{118} control lines at the white pre-pupal (WPP) stage were dissected and stained with TF marker Engrailed (En) (Fig. 4). Ovaries from the c587 > lola^{12574} RNAi line (Fig. 4A (b, c)) were much smaller than those in the c587 > W^{118} control line (Fig. 4A (a)), a result consistent with that of the adult ovaries (Fig. 3). In the ovary of c587 > W^{118} control line, TF stacks were well organized (Fig. 4A (a')), while in the ovary of c587 > lola^{12574} RNAi line, TF stacks were disordered and the number of TFs was reduced (Fig. 4A (b')). This is also consistent with the result in adult ovaries that smaller ovaries had fewer TFs (Fig. 3A (c')). In most severe cases, no TF stacks were formed in the ovary of c587 > lola^{12574} RNAi line (Fig. 4A (c')), which matches the morphology in adult ovaries that no distinguishable ovarioles were observed in the smaller ovaries (Fig. 3A (b', d')).

(Lola is involved in regulating ovarian apoptosis in the adult stage)

We observed some DNA fragments in the smaller ovaries of c587 > lola^{12574} RNAi line (Fig. 3A (c', d')) and speculate that smaller in size of ovary may be due to apoptosis in the ovarioles. To investigate this, we performed TUNEL (Terminal deoxynucleotidyl Transferase Biotin dUTP Nick End Labeling) and DAPI staining. The presence of DNA fragmentation, as depicted by green staining, was detected in 89.7% of the smaller ovaries of the c587 > lola^{12574} RNAi line (n = 29) (Fig. 5A (b', c')) but not in the ovaries of the c587 > W^{118} control line (Fig. 5A (a')). To determine whether these phenotypes were due to loss of Lola function during ovary development or in the adult stage, we utilized the well-established characteristics of Gal4 driver, which has low activity at low temperatures but increased activity at higher temperatures. The c587 > lola^{12574} and c587 > W^{118} flies were reared at 18°C from eggs to pupae with functional niches [30], and then reared at 25°C from pupae to adults to knock down *lola* expression. Ovaries from 3 to 4 days adults were dissected and stained, and the results showed that ovaries from the c587 > lola^{12574} RNAi adults under these conditions had normal size and proper number of ovarioles (Fig. 5B (b, c)) compared to those of the c587 > W^{118} control adults.
c587 upregulated and 281 genes were downregulated in the ovary of In gonad development, oogenesis and oviposition, such as mira (b) the ovarioles (c587 DEGs were also involved in metabolic process (Fig. 6C). Among the expressed genes (DEGs) were involved in reproduction and 106

This result indicated that Lola serves as a transcription factor in the RNA-Seq (with RNAs isolated from the ovaries of control and (Fig. 5B (a)). However, in 43% of ovaries from the corresponding ovaries (c587 higher magnifications of the ovary regions from the corresponding ovaries (a-h). c‘ d’ Higher magnifications of the ovary regions from the corresponding ovaries (c, d’) showing DNA fragmentations. Scale bar: 200 μm in a-h, and 100 μm in a‘-h. (Fig. 5B (a)). However, in 43% of ovaries from the c587 > lola12574 RNAi adults (n = 44), about 3–6 egg chambers showed DNA fragments during mid-oogenesis (Fig. 5B (b, c)), while in ovaries of the c587 > w1118 control adults, no more than three egg chambers showed DNA fragments (Fig. 5B (a)). These results suggest that lola represses apoptosis in Drosophila adult ovary.

Lola regulates ovarian morphology in the ovary
To better understand how lola plays such an important role in regulating ovarian development, we carried out RNA-sequencing (RNA-Seq) with RNAs isolated from the ovaries of c587 > w1118 control and c587 > lola12574 RNAi females. We identified a total of 12695 genes by RNA-Seq (Supplementary Table S1). The numbers of clean reads, expressed genes, total mapped reads and unique matches for each sample were shown in Fig. 6A. Compared with the control, a total of 433 genes have at least 1.3-fold change (q-value < 0.05) in the expression levels, of which, 152 genes were upregulated and 281 genes were downregulated in the ovary of c587 > lola12574 RNAi line (Fig. 6B, Supplementary Tables S2, S3). This result indicated that Lola serves as a transcription factor in the ovary. Gene Ontology (GO) analysis showed that 106 differentially expressed genes (DEGs) were involved in reproduction and 106 DEGs were also involved in metabolic process (Fig. 6C). Among the reproduction-related DEGs, many have been shown to play a role in gonad development, oogenesis and oviposition, such as rib, mira, p24-2 and BG642312 [31–34]. There are also 8 DEGs involved in cell growth and death (Fig. 6C), including Roc1b and tomb [35, 36].

To confirm RNA-seq data, 12 differentially expressed genes associated with reproduction and cell growth and death were selected for qRT-PCR analysis (Table 1). The results showed that expression profiles of all the select DEGs in the ovary measured by qRT-PCR were consistent with those of the RNA-seq data, with 6 DEGs (Bbd, Roc1b, CG16995, tut, tomb, and p24-2) upregulated and 6 DEGs (CG4847, Gld, CG33943, mira, lectin-30A and rib) downregulated (Fig. 6D). Taken together, these results suggest that lola regulates expression of genes involved in reproduction, cell growth and death.

Lola regulates ovarian morphology by suppressing Roc1b expression
Given that knockdown of lola in the ovary induced apoptosis, we pay attention to one DEG (Roc1b) that is related to apoptosis. It has been shown that Roc1b is required for activation of effector caspase during spermatogenesis [35], yet its role in ovarian development remains unknown. In the ovary of c587 > lola12574 RNAI line, Roc1b expression was upregulated, which may induce ovarian apoptosis. To verify whether Roc1b is responsible for the defects in ovarian morphogenesis of the c587 > lola12574 RNAI line, Roc1b was overexpressed in the ovary driven by c587-Gal4. Surprisingly, overexpression of Roc1b caused defects in ovarian morphogenesis, such as egg chamber fusion and DNA fragmentation in smaller ovaries (Fig. 7A (b, c and c‘)) or lacking ovarioles (b, d’ from the c587 > lola12574 RNAI line (n = 58). B Phenotypes of ovaries from the Tj > w1118, Tj > lola12574 and Tj > lola1205261 lines. Ovaries with normal size and number of ovarioles (e, e‘) from the Tj > w1118 control, ovaries with either one (f) or both (g) from the Tj > lola12574 RNAI line, and ovaries with one in smaller size (h) from the Tj > lola1205261 RNAI line. a‘-h’ Higher magnifications of the ovary regions from the corresponding ovaries (a-h). c‘ d’ Higher magnifications of the ovary regions from the corresponding ovaries (c, d’) showing DNA fragmentations. Scale bar: 200 μm in a-h, and 100 μm in a‘-h.

(Fig. 3B (a)). However, in 43% of ovaries from the c587 > lola12574 RNAI adults (n = 44), about 3–6 egg chambers showed DNA fragments during mid-oogenesis (Fig. 3B (b, c)), while in ovaries of the c587 > w1118 control adults, no more than three egg chambers showed DNA fragments (Fig. 3B (a)). These results suggest that lola represses apoptosis in Drosophila adult ovary.

DISCUSSION
Drosophila ovary is a model system for understanding the stem cell niche, yet the genetic mechanisms underlying establishment of the niche remain largely unknown. To date, only three transcription factors, Bab, Lmx1a and En/Inv, have been identified as essential factors for the formation or proper stacking of TFs [18, 22, 25, 26]. In this study, we characterized the function of Lola in regulation of ovary development in Drosophila. By using two independent lola RNAi lines (lola12574 and lola1205261) driven by somatic drivers c587-Gal4 and Tj-Gal4, we showed that lola is required for the formation of TFs at the time when ovarian stem cell niche is established. It has been shown that Bab is involved in the formation of TFs [18, 22, 25]. Similar to Bab protein, Lola also contains the BTB domain. Both Bab and Lola have been shown to be involved in the formation of TF. Thus, we speculate that BTB
transcription factors such as Lola and Bab may be essential for the formation of TFs.

We observed that ovarian atrophy occurred in the lola RNAi line when niches were not established normally, a result consistent with previous report that niche dysfunction may lead to tissue degeneration [37]. It has been reported that loss of lola-K resulted in blocking the developmental PCD in the late-oogenesis and disrupted the induced PCD during mid-oogenesis in response to starvation [12]. We showed that lola is related to adult ovarian apoptosis. But lola-regulated apoptosis was not due to starvation since all flies were under well-fed conditions. Also, ovarian apoptosis in lola RNAi line occurred at an earlier time (mid stage) rather than at the late stage of oogenesis and Roc1b RNAi partly rescued this phenotype, suggesting that lola control ovarian apoptosis through regulating expression of Roc1b and other genes. Given that c587 is expressed in escort cells and early follicle cells of Drosophila adults but is not expressed in the mid-oogenesis [19], lola is acting non-autonomously to promote egg chamber survival.

It has been shown that Lola-F bound to chromosomal kinase JIL-1 in yeast two hybrid assays [2]. Loss of function in JIL-1 affected female fertility and resulted in smaller ovaries [38], a morphology similar to that of lola RNAi line. However, expression of JIL-1 in the ovary of lola RNAi line did not change significantly. Further study is required to determine which Lola isoform(s) plays a role in ovarian development.

Using RNA-seq analysis, we identified 433 differentially expressed genes (DEGs) in the ovary of lola RNAi line, with 152 DEGs upregulated and 281 DEGs downregulated. Among these DEGs, we selected an apoptosis-related gene, Roc1b. It has been reported that Roc1b activates caspase activity during spermatogenesis [35], but its role in ovary development has not been well understood. We showed that overexpression of Roc1b in the ovary caused defects in ovarian morphogenesis. To our knowledge, this is the first report to demonstrate that expression of Roc1b, a downstream gene of lola, can affect ovarian development. Roc1b homolog RBX1 in human has been reported to play a role in high-grade serous ovarian cancer (HGSOC) in women [39]. Lola is a BTB-ZF (zinc finger) transcription factor that is required for stem cell maintenance in Drosophila testis [10]. Strikingly, another BTB-ZF transcription factor Promyelocytic leukemia zinc-finger (Plzf), which has a similar structure to Lola, was found to play a role in the maintenance of spermatogonial stem cells in mouse testis [40, 41]. Our findings together with the published results indicate that the reproductive mechanism is conserved from fruit flies to mammals. Therefore, investigating functions of lola in Drosophila ovary will lead to better understand the role of lola in ovary development from invertebrates to mammals.

The establishment of the stem cell niche is regulated by several signaling pathways, including target of rapamycin, insulin, ecysdysone, Hippo, and activin signaling pathways [42–45]. It remains unclear how lola plays a role in the establishment of stem cell niche, whether through one or more of the above-mentioned signaling pathways. Future studies will focus on this subject.
Fig. 5 RNAi of lola induces ovarian apoptosis. A Ovaries from the c587 > w1118 control line (a) and c587 > lola12574 RNAi line reared at 25 °C from eggs to adults (b, c) were stained with TUNEL assay for apoptotic cells (a’–c’, green), and nuclei were stained with DAPI (a’–c’, white). In a control ovary, cells were TUNEL-negative (a–a’), while in c587 > lola12574 RNAi ovaries, TUNEL-positive was observed (b–b’, c–c’). B The c587 > w1118 and c587 > lola12574 RNAi lines were reared at 18 °C from eggs to pupae and then at 25 °C from pupae to adults. Ovaries from 3-4 days c587 > w1118 and c587 > lola12574 RNAI adults were dissected and stained with DAPI. DNA fragments were detected in the ovaries of c587 > lola12574 RNAI adults (b and c, circled in yellow), but not in the ovaries of c587 > w1118 control adults (a). Scale bar: 100 µm.

Fig. 6 RNAi of lola in the ovary results in transcript alterations as assessed by RNA-seq analysis. A The numbers of total clean reads, number of genes, total mapping and unique mapping for each sample. B Volcano plot of differentially expressed genes (DEGs) in the ovary of c587 > lola12574 RNAi line relative to the c587 > w1118 control. C GO analysis of DEGs from comparison of the c587 > lola12574 RNAI and c587 > w1118 control groups. D qRT-PCR validation of select DEGs from RNA sequencing data. Significant difference was determined by the student’s t-test and indicated by *p < 0.05 and **p < 0.01.
| Relative expression | Gene symbol | Log2 Fold difference | Biological functions |
|---------------------|-------------|----------------------|----------------------|
| Up-regulated        | tut         | 1.34                 | Regulation of mitotic amplification of germ cells |
|                     | Roc1b       | 1.41                 | Involved in caspase activation during spermatogenesis |
|                     | Bbd         | 1.49                 | Involved in multicellular organism reproduction |
|                     | p24-2       | 1.71                 | Involved in post-mating oviposition |
|                     | tomb        | 1.86                 | Meiotic-arrest gene involved in spermatogenesis |
|                     | CG16995     | 2.39                 | Involved in multicellular organism reproduction |
| Down-regulated      | BG642312    | -1.62                | Regulation of oviposition |
|                     | mira        | -1.74                | Involved in oogenesis |
|                     | rib         | -1.71                | Regulation of gonad development |
|                     | Gld         | -2.1                 | Involved in sperm storage |
|                     | CG4847      | -2.48                | Involved in multicellular organism reproduction |
|                     | lectin-30A  | -4.58                | Involved in multicellular organism reproduction |

**Fig. 7.** *Lola* regulates ovarian morphogenesis by suppressing Roc1b expression. A Phenotypes of ovaries from the c587 > w¹¹¹⁸, c587 > UAS-Roc1b³⁴⁰⁴⁸, c587 > lola¹²⁵⁷⁴;Roc1b¹³⁰⁶⁷, c587 > lola¹²⁵⁷⁴;GFP and c587 > Roc1b¹³⁰⁶⁷ lines. a Ovaries with normal size and number of ovarioles from the c587 > w¹¹¹⁸ control. b, c Overexpression of Roc1b resulted in one smaller ovary with fewer ovarioles (b) or a pair of smaller ovaries with fused egg chambers (c), which mimic the phenotypes of lola¹²⁵⁷⁴ RNAi. d Knockdown of Roc1b in the c587 > lola¹²⁵⁷⁴ RNAi line rescued defects in ovarian morphogenesis. e, f Knockdown of GFP in the c587 > lola¹²⁵⁷⁴ RNAi line resulted in one smaller ovary with fewer ovarioles (e) or a pair of smaller ovaries with fused egg chambers (f). g Ovaries with normal size and number of ovarioles from the c587 > Roc1b¹³⁰⁶⁷. c' Higher magnification of the ovary region from c. B Knockdown expression of Roc1b in flies (c587 > Roc1b¹³⁰⁶⁷) did not have an effect on female fertility, while knockdown expression of Roc1b in the c587 > lola¹²⁵⁷⁴ RNAi line increased female fertility. C Expression of Roc1b in the ovaries of the c587 > Roc1b¹³⁰⁶⁷ and c587 > w¹¹¹⁸ control lines. Significant difference was determined by the student’s t-test and indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns for non-significant. Scale bar: 100 µm in a–g, 300 µm in c'.
Diego, CA, USA). The single-stranded DNA was used to circularize the single-stranded DNA to obtain a single-stranded ampli (Transgene, Beijing, China); (3) the ds-cDNAs were subjected to traditional EasyScript purification with poly(A) tails; (2) the Trizol (Invitrogen, CA, USA) following the recommendations of the manufacturer. Total RNA was used in the following procedures: (1) oligo-dT magnetic beads was used to purify mRNA; (2) the purified mRNA was fragmented and reverse transcribed to synthesize double-stranded cDNAs (ds-cDNAs) using EasyScript RT/RI Enzyme Mix (Transgene, Beijing, China); (3) the ds-cDNAs were subjected to traditional processing that included ligation of indexed Illumina adapters and amplification using limited-cycle PCR. (4) The ds-cDNAs PCR products were heat-denatured to form single strands, and then a bridge primer was used to circularize the single-stranded DNA to obtain a single-stranded circular DNA library; (5) sequencing was performed at the Beijing Genomics Institute (BGI, Shenzhen, China) using an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA).

Bioinformatics analysis of RNA-seq data

The transcript abundances in this study were determined using Fragments Per Kilobase of Million mapped reads (FPKM) values. Differentially expressed genes (DEGs) were identified based on a log2 fold-change > 1.3 (or log2 fold-change < –1.3) and a p < 0.05 with four biological replicates. Volcano plot was obtained to display visually the distribution of fold-change and fold-change values of DEGs between two groups. Gene Ontology (GO) enrichment analysis of DEGs was performed by using the GOseq R package.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from 3-day-old c587 > lolaΔ2574 RNAi and c587 > w1118 control flies in this study. Ovaries from 80 c587 > lolaΔ2574 RNAi females and 40 c587 > w1118 females were dissected in PBS. Total RNA was extracted with Trizol (Invitrogen, CA, USA) following the recommendations of the manufacturer. We used 3-day-old c587 > lolaΔ2574 RNAi and c587 > w1118 control female ovaries using Trizol reagent (Invitrogen). The first-strand cDNA was synthesized from 2 μg of total RNA using EasyScript first-strand cDNA synthesis SuperMix (Transgene, Beijing, China). qRT-PCR was performed with a TransStart Tip Green qPCR SuperMix (Transgene). The qRT-PCR experiments were conducted using a CFX connect™ real-time system (BioRad, CA, USA), as described previously [48]. The relative expression of the gene was calibrated against the reference gene rp49 using 2^ΔCT (ΔCT = CT target gene–CT rp49). The primers used in this study are shown in Supplementary Table S4.

Statistical analysis

Three biological replicates and three technical replicates for each biological replicate were performed. The significant difference was determined by the student’s t-test or by one-way ANOVA followed by a post hoc Tukey’s honestly significant difference (HSD) test using GraphPad.

DATA AVAILABILITY

All datasets generated and analyzed during this study are included in this published article and its Supplementary Information files. Additional data are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

TZ participated in experimental design, performed most experiments, analyzed the data, and wrote the manuscript; YX and BH helped dissect ovaries; M-JR and XD provided analytical tools; Y-FW helped write the manuscript; YL and X-QY participated in study conception and experimental design, performed data analysis, supervised the study, and wrote the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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