Juvenile hormone signaling promotes ovulation and maintains egg shape by inducing expression of extracellular matrix genes

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It is well documented that the juvenile hormone (JH) can function as a gonadotropic hormone that stimulates vitellogenesis by activating the production and uptake of vitellogenin in insects. Here, we describe a phenotype associated with mutations in the *Drosophila* JH receptor genes, *Met* and *Gce*: the accumulation of mature eggs with reduced egg length in the ovary. JH signaling is mainly activated in ovarian muscle cells and induces *laminin* gene expression in these cells. Meanwhile, JH signaling induces *collagen IV* gene expression in the adult fat body, from which collagen IV is secreted and deposited onto the ovarian muscles. Laminin locally and collagen IV remotely contribute to the assembly of ovarian muscle extracellular matrix (ECM); moreover, the ECM components are indispensable for ovarian muscle contraction. Furthermore, ovarian muscle contraction externally generates a mechanical force to promote ovulation and maintain egg shape. This work reveals an important mechanism for JH-regulated insect reproduction.

laminin | collagen IV | visceral muscle | ovarian contraction | egg chamber elongation

Reproduction is a fundamental feature of all known life, and the fruit fly, *Drosophila melanogaster*, is an ideal invertebrate model for reproduction biology studies. *Drosophila* females have a pair of ovaries, and each ovary contains ∼16 ovarioles. There are two types of ovarian visceral muscles: the peritoneal sheath muscle covers each intact ovary, and the epithelial sheath muscle surrounds each ovariole. Each kind of visceral muscle is covered by a basement membrane (BM: a specialized extracellular matrix [ECM]) at both sides. The peritoneal sheath muscle has a mesh-like morphology, while the epithelial sheath muscle is circular (1, 2). An ovariole harbors the germarium at its anterior tip, followed by an array of egg chambers of increasing developmental stage. Each egg arises from an egg chamber composed of 16 germ cells, including 15 nurse cells and 1 oocyte, and surrounded by a monolayer of epithelial follicle cells (FCs) (3). Largely based on morphology, egg chambers are divided into 14 developmental stages. Stage one buds from the germarium, and stage 14 is the mature egg (4). The egg chamber is initially spherical and progressively becomes elliptical during oogenesis (Fig. 1*A*). FCs undergo a collective migration perpendicular to the anterior–posterior (A-P) axis, which causes the egg chamber to rotate within its surrounding BM, resulting in egg chamber elongation and egg shape formation (5). The egg chamber slowly rotates during stages 1 to 4, while it rotates much faster and begins to elongate during stages 5 to 8 because of the force of FC "molecular corset" (6, 7). During stages 9 and 10, the egg chamber continues to elongate because of oscillating, myosin-mediated contraction, and the oocyte grows dramatically because of the uptake of yolk proteins (i.e., vitellogenin, Vg). After stage 10, growth stops, and the active phase of egg chamber elongation is complete. During stage 11, the nurse cells transfer their cytoplasm to the oocyte, which rapidly expands (8, 9). Later on, the mature egg leaves from the ovary, enters the oviduct, and activates when transported into the uterus, where fertilization occurs (10, 11).

In recent years, significant progress has been made regarding the mechanisms of egg shape maintenance and ovulation induction in *Drosophila*. The FC collective migration drives egg chamber rotation, promoting the global alignment of contractile actin bundles to establish the actin planar polarity (6, 7). Egg chamber rotation also promotes the formation of a planarly polarized, fibril-like BM structure, which is maintained by the ECM components, including collagen IV, laminin, nidogen and perlecan, as well as the cell–ECM-interacting molecules [i.e., integrins (1, 7, 12)]. A positive regulatory loop accelerates the formation of planarly polarized actin bundles and fibrillar BM, which act as the "molecular corset" that resists the germ cells' expansive growth. Thus, the "molecular corset" biases egg chamber growth along the A-P axis, determining the elongated and elliptical egg shape (5).

Significance

The juvenile hormone (JH) plays a key role in regulating insect reproduction. Previous studies have demonstrated how JH activates vitellogenin production in the fat body and stimulates vitellogenin uptake by maturing oocytes. Our studies show that, in *Drosophila*, JH signaling induces *laminin* expression in ovarian muscle cells and *collagen IV* expression in the adult fat body. Laminin locally and collagen IV remotely contribute to the assembly of ovarian muscle extracellular matrix (ECM). The ECM components are indispensable for ovarian muscle contraction that externally generates a mechanical force to promote ovulation and maintain egg shape. This study reveals an essential role of JH in the regulation of insect reproduction.

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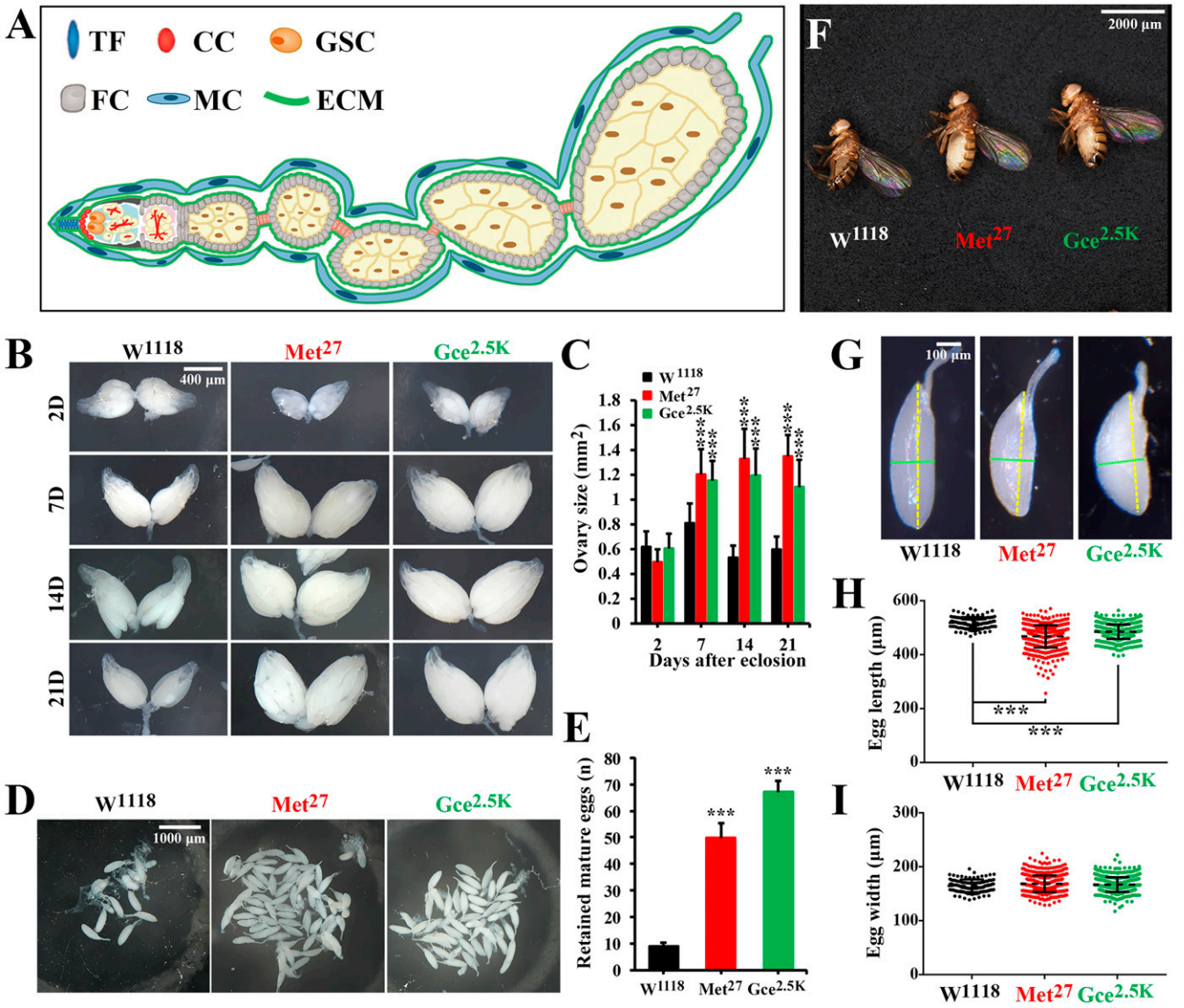

Fig. 1. Ovulation deficiency and abnormal egg shape in unmated *Met*^{27} and *Gce*$^{2.5K}$. (*A*) Illustration of an ovariole: the germarium at its anterior tip followed by an array of egg chambers of increasing developmental stage. ECM is located around the ovarian muscle cells and egg chamber. TF: terminal filament, CC: cap cells, GSC: germline stem cells, FC: follicular cells, and MC: muscle cells. (*B*) Images of *W*^{1118}, *Met*^{27}, and *Gce*$^{2.5K}$ ovaries on d2AAE, d7AEE, d14AAE, and d21AEE. (*C*) Quantification of ovary sizes shown in *B*, ***$P < 0.001$. (*D*) Images of retained mature eggs from *W*^{1118}, *Met*^{27}, and *Gce*$^{2.5K}$ ovaries on d14AAE. (*E*) Quantification of the retained mature eggs in each pair of ovaries, ***$P < 0.001$. (*F*) Images of *W*^{1118}, *Met*^{27}, and *Gce*$^{2.5K}$ virgin females on d14AAE, showing expanded abdomens in the mutants. (*G*) Representative images showing that mature egg shape is changed in *Met*^{27} and *Gce*$^{2.5K}$. (*H* and *I*) Quantification of length (*H*) and width (*I*) of eggs shown in *G*, ***$P < 0.001$.

As an essential step in reproduction, ovulation is regulated by several signaling molecules, including octopamine, 20-hydroxyecdysone (20E), and ovulin that might cross talk with one another (11, 13, 14). It has been hypothesized that ovulation involves the rhythmic contraction of ovarian muscles (15). The formation of dysfunctional epithelial sheath muscle, due to the mutation of the irre cell recognition module (IRM) genes or reduction of FGF or Wnt signaling, leads to abnormal ovarian muscle contractions, which fail to provide mechanical power (16–18). Interestingly, it has been reported that ovarian muscle contraction is involved in yolk protein uptake, oocyte growth, egg chamber elongation, and egg shape. During these processes, the BM component laminin W plays a critical role (19). Nevertheless, little is known about how egg shape and ovulation are coordinately regulated and whether ovarian muscles are involved in this coordination.

The juvenile hormone (JH) maintains juvenile characters and prevents metamorphosis by antagonizing 20E actions. JH also regulates female reproduction, while the mechanism of JH action in reproduction varies broadly (20–23). In *Drosophila*, there are two redundant JH intracellular receptors, Methoprene-tolerant (Met) and germ cell-expressed (Gce) (24–30). Upon JH binding, Met/Gce enters the nucleus and binds to E-box–like motifs in promoter regions of JH primary response genes to induce gene expression (31–33). *Krüppel homolog 1* (*Kr-h1*), a crucial JH primary response gene, acts as an anti-metamorphic factor that antagonizes 20E actions to prevent metamorphosis (34, 35). As shown in a number of insects, JH also acts as a gonadotropic hormone to stimulate vitellogenesis through activating the production and uptake of Vg (22). JH intracellular signaling (hereafter referred to as JH signaling if not mentioned otherwise) stimulates Vg production in the fat

body by inducing *Vg* expression, as well as activating DNA replication and polyploidization of fat body cells (36–39). Moreover, JH induces Vg uptake in maturing oocytes by enlarging FC intercellular spaces (follicular patency) via a putative membrane receptor, which is likely a receptor tyrosine kinase (40–43). In this study, we have discovered that, in addition to activating vitellogenesis, JH signaling plays an important role in female reproduction: It induces ECM gene expression in ovarian muscle cells and the adult fat body. The ECM components are indispensable for ovarian muscle contraction that externally generates a mechanical force to promote ovulation and maintain egg shape in *Drosophila*.

Results

Reduction of JH Signaling Causes Ovulation Deficiency and Abnormal Egg Shape. We have previously shown that the reduction in JH levels in *Drosophila* females, by the genetic ablation of the JH-

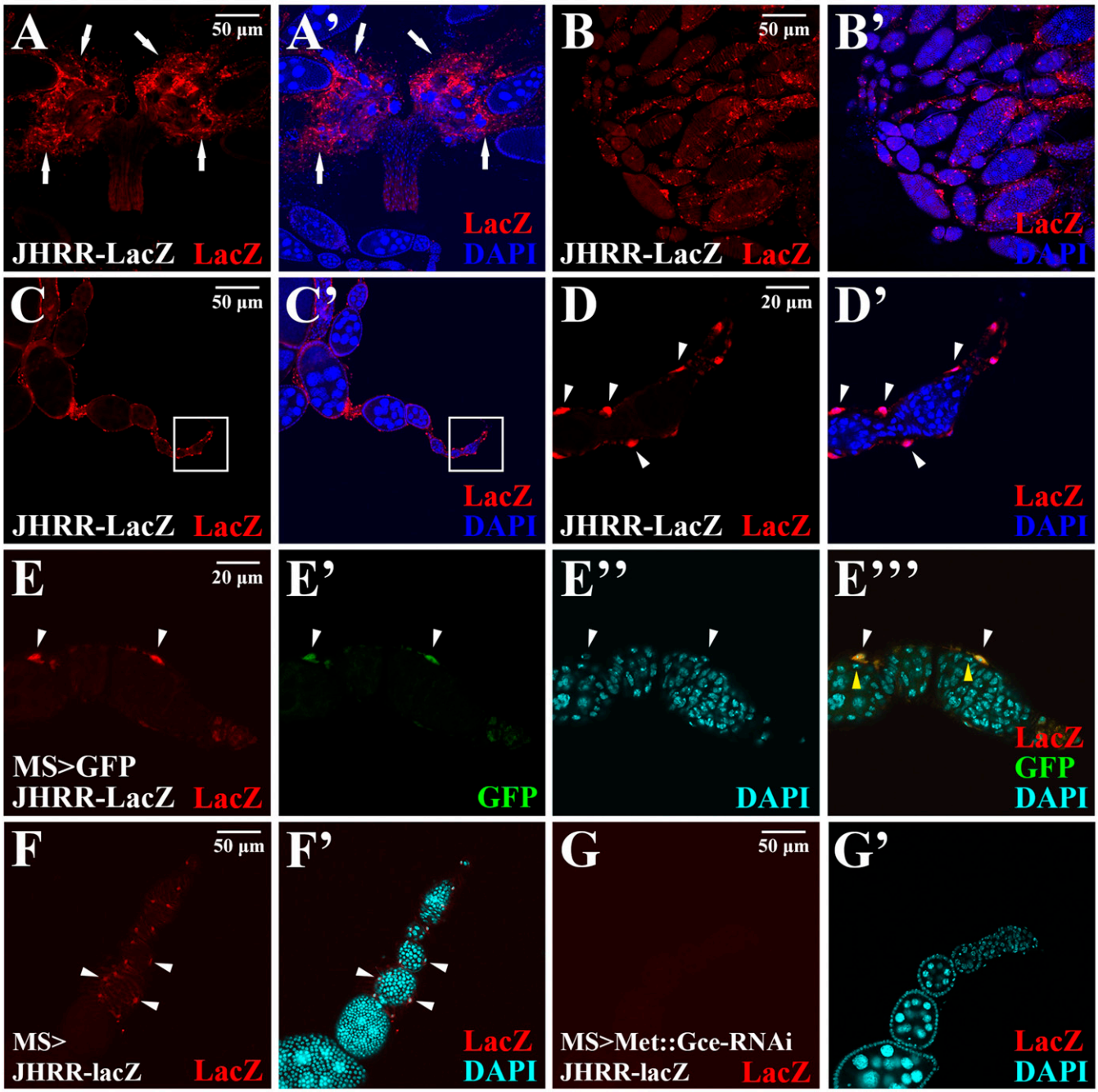

Fig. 2. JH signaling is exclusively activated in ovarian muscle cells. (*A–D'*) Confocal images of *JHRR-lacZ* in ovaries stained with LacZ antibody (red) and DAPI (blue), showing that LacZ staining is specifically located at ovarian peritoneal sheath cells (*A* and *A'*, white arrows) and epithelial sheath cells (*B–D'*, white arrowheads). (*D* and *D'*) The enlarged images of the region defined by white boxes in *C* and *C'*. (*E–E'''*) Confocal images of ovaries labeled by LacZ antibody staining (red), DAPI (blue), and GFP (green), showing that *JHRR-LacZ* coexpressed with *MS-Gal4 > UAS-GFP* in ovarian muscle cells. The white arrowheads point to the epithelial sheath muscle cell and the yellow arrowheads point to the FC. (*F–G'*) *JHRR-LacZ* expression is suppressed by *UAS-Met/Gce* RNAi driven by *MS-Gal4* (*G* and *G'*) compared with the control (*F* and *F'*). LacZ is shown in red. Cell nuclei labeled with DAPI (blue). The white arrowheads point to the epithelial sheath muscle cell.

producing organ corpus allatum or mutation of the key regulatory enzyme gene *JH acid methyltransferase* (*Jhamt*) in the JH biosynthesis pathway, decreased fecundity with reduced oviposition (egg laying) and decreased ovary size (30, 44). These reproductive deficiencies have been suggested to result from decreases of JH-induced Vg production in the fat body and Vg uptake by the oocytes (21). In agreement with the previous findings in other insects (21, 22), the topical application of the JH analog methoprene partially restores fecundity and ovary size in the *Jhamt* mutant, $Jhamt^2$ (*SI Appendix*, Fig. S1 *A* and *B*). Moreover, methoprene treatment in $Jhamt^2$ also induced *Vg* expression in the adult fat body and follicular patency in maturing oocytes (*SI Appendix*, Fig. S1 *C–D'*) (45, 46). Similar to corpus allatum ablation and *Jhamt* mutation, the null mutant of a single-JH receptor gene, Met^{27} or $Gce^{2.5K}$, showed decreased fecundity with reduced oviposition (24, 47).

To test whether the decreased fecundity in Met^{27} or $Gce^{2.5K}$ is also accompanied by reduced ovarian size in response to attenuated JH signaling, we first measured the ovary size of unmated Met^{27} or $Gce^{2.5K}$. We surprisingly found that the result was the opposite. The ovary size was comparable among Met^{27}, $Gce^{2.5K}$, and the wild-type control W^{1118} on day 2 after adult eclosion (d2AAE). However, the Met^{27} or $Gce^{2.5K}$ ovaries became significantly larger than those of W^{1118} flies on d7AAE, d14AEE, and d21AAE (Fig. 1 *B* and *C*). The ovarian enlargement in Met^{27} or $Gce^{2.5K}$ on d14AAE was caused by the accumulation of fully developed mature eggs in the ovarioles, showing a severe ovulation deficiency. In comparison, a pair of wild-type ovaries contained ~10 mature eggs on d14AAE, while a pair of ovaries in Met^{27} or $Gce^{2.5K}$ retained 50 to 70 mature eggs, resulting in larger ovaries and more expanded abdomen (Fig. 1 *D–F*). We also examined whether ovulation was affected in the mated Met^{27} or $Gce^{2.5K}$. The mated Met^{27} exhibited similar but less significant phenotypic defects compared to the unmated Met^{27}, while the mated $Gce^{2.5K}$ showed weak phenotypic defects (*SI Appendix*, Fig. S2).

Interestingly, egg length, but not egg width, of the mature eggs was reduced by ~10% in unmated Met^{27} or $Gce^{2.5K}$ (Fig. 1 *G–I*). Taken together, we described a phenotype associated with Met^{27} or $Gce^{2.5K}$: the accumulation of mature eggs with reduced egg length in the ovary. The results revealed a previously unidentified JH action in *Drosophila* reproductive development.

JH Signaling Pathway Is Mainly Activated in Ovarian Muscle Cells. *JHRR-LacZ* is an activity indicator of JH signaling, which is based on the JH response region (JHRR) of the *Drosophila Kr-h1* promoter and recapitulates the responsiveness of *Kr-h1* to JH and Met/Gce (31, 34). To determine whether JH signaling functions directly on the ovary and which types of cells in the ovary respond to JH signaling, we monitored the expression pattern of *JHRR-LacZ*. Surprisingly, *JHRR-LacZ* was exclusively expressed in the peritoneal sheath muscle cells and the epithelial sheath muscle cells (Fig. 2 *A–D'*). In the subsequent experiments, we only examined muscle cells in the epithelial sheath, which are referred to as the ovarian muscle cells in this paper. To confirm this finding, we coexpressed *JHRR-LacZ* with GFP driven by two muscle-specific Gal4 lines: *MS-Gal4* and *Htl-Gal4* (16). Importantly, LacZ staining was always colocalized with GFP in the muscle cells, but not any other types of cells in the ovary (Fig. 2 *E–E'''* and *SI Appendix*, Fig. S3 *A–A'''*). To determine that the expression of *JHRR-LacZ* in ovarian muscle cells is regulated by JH signaling, we performed RNA interference (RNAi) experiments to deplete the expression of *Met*/*Gce* only in the muscle using *MS-Gal4* or *Htl-Gal4*. Crucially, RNAi knockdown of *Met*/*Gce* in ovarian muscle cells significantly reduced LacZ staining (Fig. 2 *F–G'* and *SI Appendix*, Fig. S3 *B–C'*). In contrast, *JHRR-LacZ* staining revealed no coexpression with GFP that is driven by an FC-specific Gal4: *109–30-Gal4* (48) (*SI Appendix*, Fig. S3 *D–E'''*). These results collectively demonstrate that, in the ovary, JH signaling is exclusively activated in ovarian muscle cells.

JH Stimulates Ovarian Muscle Contraction to Promote Ovulation. We then asked whether and how the reduction of JH signaling in ovarian muscle cells affects fecundity. To address this question, we first depleted the expression of *Met*/*Gce* or *Kr-h1* using *MS-Gal4*. The reduction of JH signaling in ovarian muscle cells significantly decreased fecundity, including reduced oviposition (Fig. 3*A*), an increased ovary size (Fig. 3*B* and *SI Appendix*, Fig. S4 *A–C*), accumulation of mature eggs (Fig. 3*C* and *SI Appendix*, Fig. S4 *D–F*), and an enlarged abdomen (Fig. 3*D*).We also used *Htl-Gal4* and *Mef2-Gal4* (19) to deplete the expression of *Met*/*Gce* or *Kr-h1*. In these three Gal4 lines, *MS-Gal4* and *Htl-Gal4* are more specific in the ovarian muscle, while *Mef2-Gal4* shows the highest expression strengthen but at a relatively low specificity (*SI Appendix*, Fig. S5). Nevertheless, using *Mef2-Gal4* to deplete JH signaling decreased fecundity more significantly than the other two Gal4 lines (*SI Appendix*, Fig. S6). Conclusively, the muscle-specific depletion of JH signaling results in fecundity defects like those observed in Met^{27} or $Gce^{2.5K}$ (Fig. 1) (24, 47). In addition, although *Met* overexpression in Met^{27} muscle cells caused lethality, the fecundity defects in $Gce^{2.5K}$ were able to be partially rescued by *Gce* overexpression using *MS-Gal4* (*SI Appendix*, Fig. S7), supporting the conclusion that JH signaling in ovarian muscle cells is essential for fecundity.

To investigate whether JH signaling in muscle cells facilitates ovulation through promoting ovarian muscle contraction, ovaries from different genotypes were cultured in vitro for live imaging, and muscle contraction was recorded and manually counted. Rapid, smooth, and energetic muscle contraction was observed in the cultured ovaries isolated from the control flies, *MS>* and W^{1118}, showing 20 to 30 contractions/min on d14AAE and d7AAE, respectively. By contrast, slow, uncoordinated, and weak muscle contraction (5 to 10 contractions/min) occurred in the cultured ovaries of the flies in which *Met*/*Gce* or *Kr-h1* was depleted in the muscle cells by RNAi as well as Met^{27} or $Gce^{2.5K}$ (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S6 *G* and *N* and Movies S1–S4). To complement the loss-of-function studies, we cultured the $Jhamt^2$ ovaries in vitro for 2 d after topical application of acetone or methoprene on d1AAE. Live imaging results showed that methoprene treatment doubled ovarian muscle contraction in the $Jhamt^2$ ovaries (*SI Appendix*, Fig. S1*E* and Movie S5).

The sarcomere is the basic contractile unit in visceral muscle cells, which is composed of thin filaments of actin and thick filaments of myosin. During muscle contraction, myosin pulls on the actin, shortening the distance between the Z-discs. Weakened muscle contraction is usually associated with a disorganized sarcomere structure, leading to elongated sarcomeres (2, 19). Thus, we examined the sarcomere structure of the ovarian muscle cells through integrin staining, which labels sarcomere Z-discs. The average distance between the Z-discs in ovarian muscles in the control flies was ~6 μm, while it was 7 to 8 μm in the flies in which JH signaling was reduced by either RNAi or mutation (Fig. 3 *G–J*). Because the two types of ovarian visceral muscles cover both ovary and ovariole, we assume that JH signaling stimulates the ovarian muscle contraction that generates a mechanical power to promote ovulation.

JH Signaling in Ovarian Muscles Affects Egg Chamber Elongation and Egg Shape. Similar to the phenotypic changes observed in Met^{27} or $Gce^{2.5K}$ (Fig. 1 *G–I*), egg length, but not egg width, of mature eggs, was reduced by ~10% in the flies in which *Met*/*Gce* or *Kr-h1* was depleted in the muscle cells (Fig. 4 *A–C*). These results are in agreement with the previous finding that abnormal muscle contraction reduces egg length and changes egg shape (19).

The FC collective migration and the "molecular corset" mechanism are well known for controlling egg chamber elongation

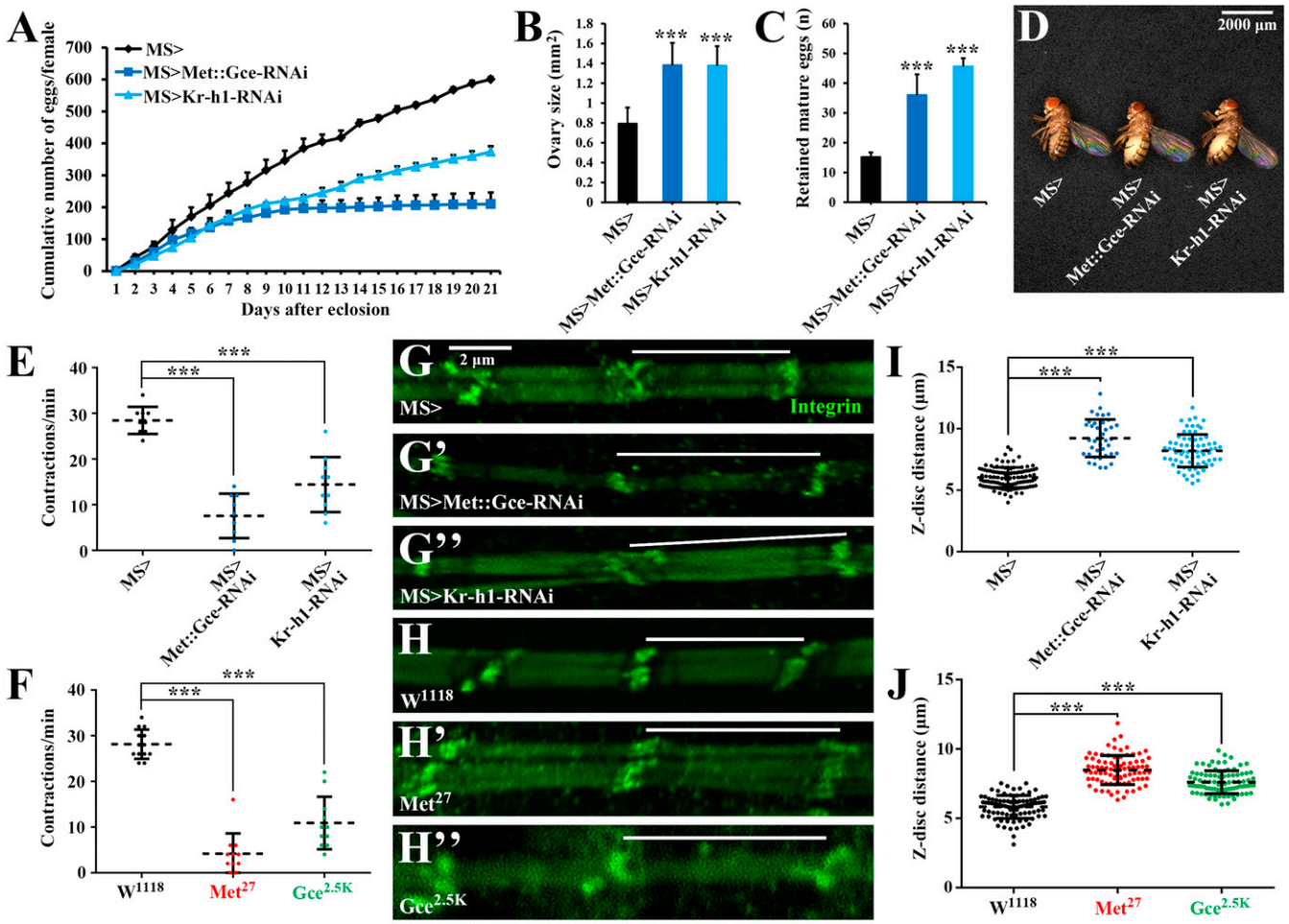

Fig. 3. Suppression of JH signaling in the ovarian muscle cells results in ovulation deficiency and muscle dysfunction. (*A–D*) The expression of *Met*/*Gce* or *Kr-h1* was depleted by RNAi using *MS-Gal4*. The reduction of JH signaling in the ovarian muscle cells significantly decreased fecundity, including reduced oviposition (*A*), increased ovarian size (*B*), accumulated mature eggs (*C*), and enlarged abdomen (*D*). (*E* and *F*) Ovarian muscle contraction rates in *UAS-Met*/*Gce* RNAi or *UAS-Kr-h1* RNAi flies driven by *MS-Gal4* (*E*) and *Met* or *Gce* mutants (*F*). Student's *t* test: ***$P < 0.001$. (*G–J*) Sarcomere structure of stage 14 egg chambers of *UAS-Met*/*Gce* RNAi or *UAS-Kr-h1* RNAi flies driven by *MS-Gal4* (*G–G''*) and *Met* or *Gce* mutants (*H–H''*). (*I* and *J*) Quantification of Z-disk distance. Student's *t* test: ***$P < 0.001$.

and egg shape formation (5–7, 9). Since the epithelial sheath muscle surrounds egg chambers in each ovariole, we hypothesize that ovarian muscles might also affect egg chamber elongation. To test this hypothesis, we measured the rate of FC collective migration that drives egg chamber rotation and the planar polarity of FC actin bundles that reflects the "molecular corset." The FC migration rate was reduced by about a half in the JH signaling-deficient flies (Fig. 4 *D–G* and Movies S6–S11), in which the FC actin planar polarity was also disturbed (*SI Appendix*, Fig. S8). Thus, JH signaling elongates egg chamber and maintains egg shape by causing ovarian muscle contraction and generating mechanical power.

JH-Induced *Laminin* Expression in Ovarian Muscle Cells Locally Contributes to Ovarian Muscle ECM Assembly and Function. It has been reported that the depletion of *laminin W* in the muscle cells decreases ovarian muscle contraction and egg length (19). We then examined whether JH signaling might induce *laminin* expression in the ovarian muscle cells to regulate ovulation and egg shape. Real-time qPCR analysis revealed that, compared to the control *MS>*, the expression levels of several *laminin* genes (*LanA*, *LanB1*, and *LanB2*) were significantly decreased in *MS > Met/Gce-RNAi* and *MS > Kr-h1-RNAi* ovaries (Fig. 5*A*). By contrast, the topical application of methoprene on *Jhamt*^{2} flies significantly induced the expression of the three *laminin* genes in the ovary (Fig. 5*B*).

Therefore, we further examined whether the depletion of JH-induced *laminin* expression in ovarian muscle cells phenocopied the defects caused by the reduction of JH signaling. When RNAi was used to individually deplete the three *laminin* genes in muscle cells, fecundity was decreased, which was accompanied by reduced oviposition (Fig. 5*C*), an increased ovarian size (Fig. 5*D* and *SI Appendix*, Fig. S9 *A–D*), accumulated mature eggs (Fig. 5*E* and *SI Appendix* Fig. S9 *E–H*), and an enlarged abdomen (Fig. 5*F*). Moreover, RNAi knockdown of the three *laminin* genes reduced ovarian muscle contraction (Fig. 5*G* and Movie S12) and sarcomere structure (Fig. 5*H* and *SI Appendix*, Fig. S9 *I–I'''*). Furthermore, egg length, but not egg width, was also reduced in the laminin-depleted flies (Fig. 5 *I* and *J* and *SI Appendix*, Fig. S9 *J–J'''*). Interestingly, although the FC migration rate was slightly reduced (Fig. 5*K* and *SI Appendix*, Fig. S9 *K–K'''* and Movies S13–S16), the FC actin planar polarity was not affected in these flies (*SI Appendix*, Fig. S9 *L–L'''*). The composite experimental data show that JH signaling induces *laminin* expression in ovarian muscle cells. The muscle ECM component laminin locally contributes to ovarian muscle contraction in promoting ovulation and maintaining egg shape, and this effect is indispensable.

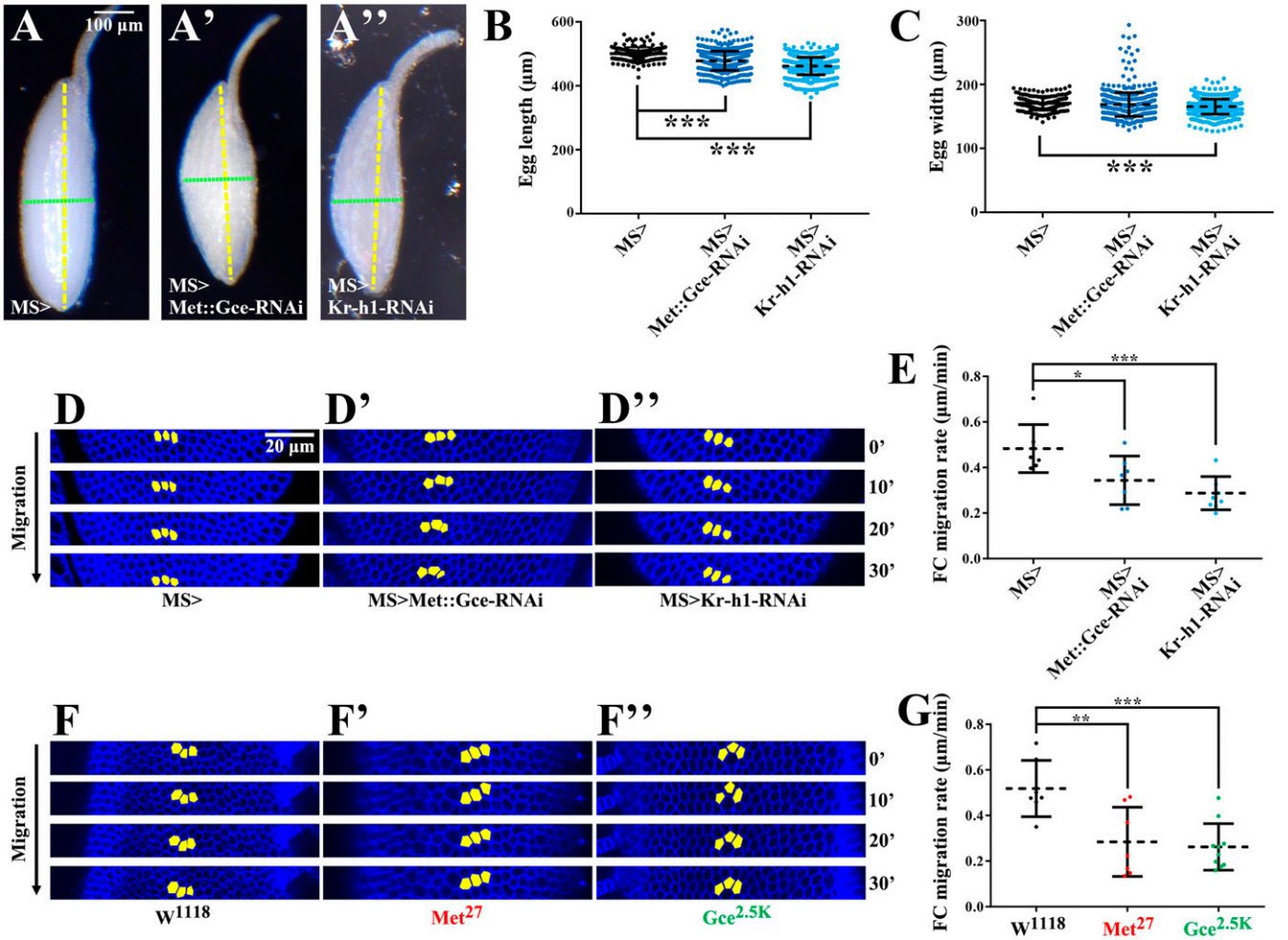

Fig. 4. Suppression of JH signaling in the ovarian muscle cells affects egg shape and FC migration rate. (*A–A''*) Egg shape changes in *UAS-Met/Gce* RNAi or *UAS-Kr-h1* RNAi flies driven by *MS-Gal4*. (*B* and *C*) Quantification of length (*B*) and width (*C*) of eggs shown in *A–A''*. (*D–E*) Changes of FC migration rate in *UAS-Met/Gce* RNAi or *UAS-Kr-h1* RNAi flies driven by *MS-Gal4* (*D–D''*). (*E*) Quantification of Z-disk distance. Student's *t* test: $*P < 0.05$ and $***P < 0.001$. (*F–G*) Changes of FC migration rate in *Met* or *Gce* mutants (*F–F''*). (*G*) Quantification of Z-disk distance. Student's *t* test: $**P < 0.01$ and $***P < 0.001$.

JH-Induced *collagen IV* Expression in Adult Fat Body Remotely Contributes to Ovarian Muscle ECM Assembly and Function. Laminin and collagen IV are the two major ECM components (1, 7, 12). Since JH signaling induces *laminin* expression to function in the ovarian muscle cells, it is likely that collagen IV should be involved in this event. However, *collagen IV* (*Col4a1* or *Vkg*) is not expressed in ovarian muscles but mainly in the adult fat body and hemolytic cells. Collagen IV is released into the hemolymph from the adult fat body and then deposited onto the ovarian muscles for ECM assembly (49, 50).

In accordance with the results that JH was able to induce *Vg* expression in *Drosophila* (*SI Appendix*, Fig. S1*C*) (45), *JHRR-LacZ* was expressed in the adult fat body (*SI Appendix*, Fig. S10 *A–A''*). We also detected the expression of *UAS-GFP* driven by *Met-Gal4* and *Gce-Gal4* (51), showing extensive GFP intensity in the adult fat body (*SI Appendix*, Fig. S10 *B–C''*). To examine whether the expression of *JHRR-LacZ* in the adult fat body is regulated by JH signaling, we performed RNAi experiments to deplete the expression of *Met*/*Gce* using *Lpp-Gal4*, which is highly expressed in the adult fat body (49, 50). As expected, RNAi knockdown of *Met*/*Gce* significantly reduced the LacZ staining (Fig. 6 *A–C*). These results together demonstrated that, as in other insects (21, 22), the adult fat body is a target of JH in *Drosophila*.

Then, we investigated whether JH signaling might induce *collagen IV* expression in the adult fat body to function as ECM components for the ovarian muscles. *Cg > GFP* is expressed under the control of regulatory sequences common to *Col4a1* and *Vkg* (49, 50). Compared to the control *Cg > GFP*, the GFP intensity significantly decreased in the adult fat body of either Met^{27} or $Gce^{2.5k}$ (Fig. 6 *D–G*). The expression levels of *Col4a1* and *Vkg* in the adult fat body strongly decreased in the mutant of either JH receptor gene (Fig. 6*H*). *Vkg-GFP* is a functional GFP trap fusion to Vkg (49, 50). Vkg-GFP is observed in the ovarian muscles. Meanwhile, it is significantly reduced in either Met^{27} or $Gce^{2.5k}$ (Fig. 6 *I–L*), indicating that the JH-induced collagen IV in the adult fat body remotely contributes to the integrity of ovarian muscle ECM. Compared to the control fly *Lpp > Vkg-GFP*, RNAi knockdown of *Met*/*Gce* or *Kr-h1* in the adult fat body significantly reduced its GFP intensity (Fig. 6 *M–P*). Likewise, RNAi knockdown of *Met*/*Gce* or *Kr-h1* in the adult fat body down-regulated the expression of *Col4a1* and *Vkg* (Fig. 6*Q*). In contrast with the above loss-of-function experiments, the topical application of methoprene to the $Jhamt^{2}$ flies significantly induced the expression of *Col4a1* and *Vkg* (Fig. 6*R*). The composite data confirm that JH signaling induces *collagen IV* expression in the adult fat body, and fat, body-derived collagen IV remotely contributes to the assembly of ovarian muscle ECM.

Finally, we tested whether JH signaling and the JH-induced *collagen IV* expression in the adult fat body are required for ovarian muscle ECM function by regulating ovulation and egg

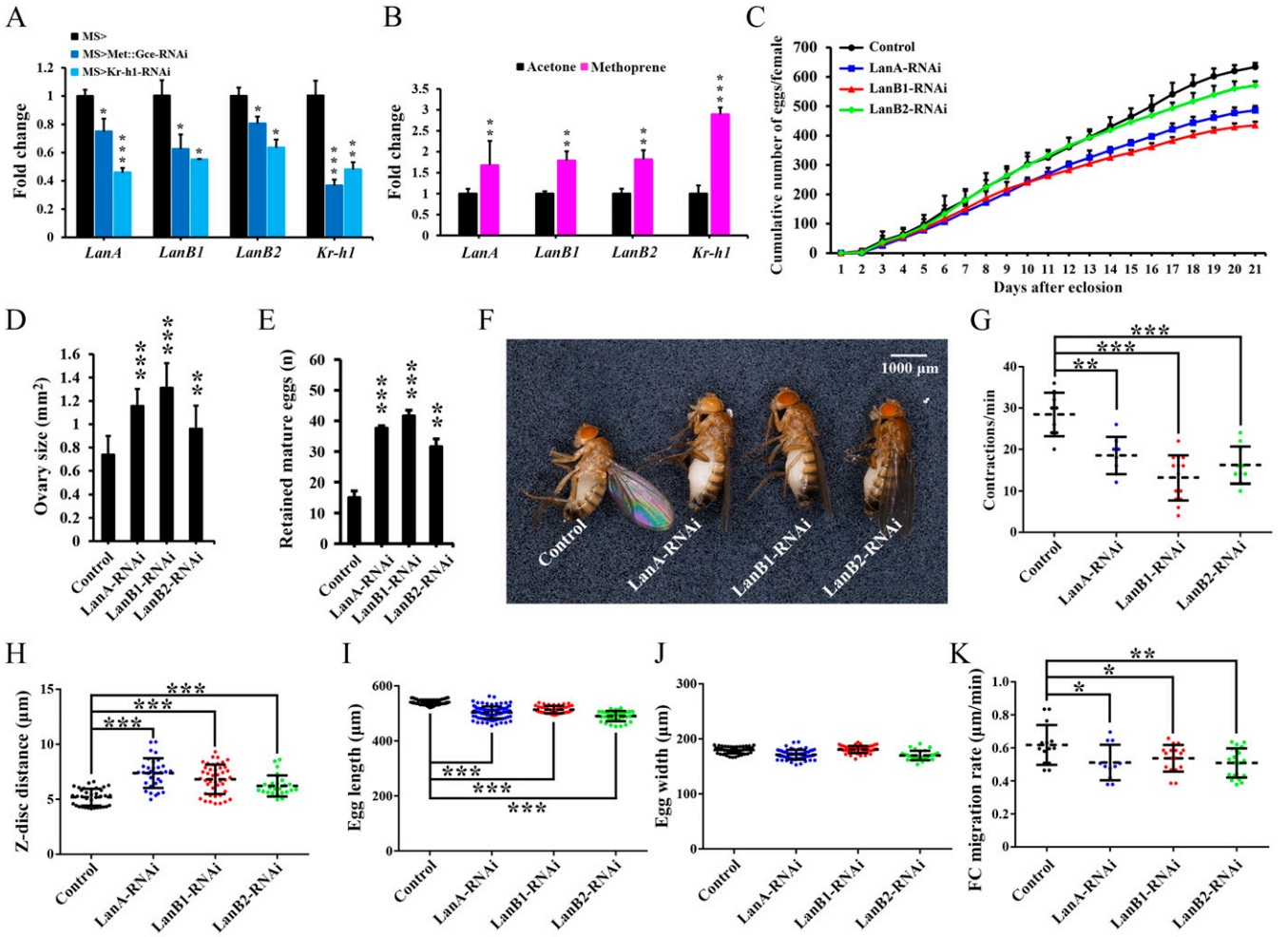

Fig. 5. Down-regulation of *laminin* (*Lan*) genes in ovarian muscle cells using *MS-Gal4* inhibits ovulation and disturbs egg shape. (*A*) qPCR analysis of JH-regulated *Lan* genes (*LanA*, *LanB1*, and *LanB2*) in the ovaries of control, *Met*/*Gce* RNAi, and *Kr-h1* RNAi flies. (*B*) qPCR analysis of JH-regulated *Lan* genes in the ovaries of $Jhamt^2$ females treated with methoprene or acetone control. (*C*–*F*) RNAi depletion of each *Lan* gene in ovarian muscle cells significantly decreased fecundity, including reduced oviposition (*C*), increased ovarian size (*D*), accumulated mature eggs (*E*), and enlarged abdomen (*F*). (*G* and *H*) RNAi depletion of each *Lan* gene in ovarian muscle cells resulted in reduced muscle contraction (*G*) and elongated sarcomere (*H*). (*I* and *J*) RNAi depletion of each *Lan* gene in ovarian muscle cells reduced egg length (*I*) but not egg width (*J*). (*K*) RNAi depletion of each *Lan* gene in ovarian muscle cells reduced the FC migration rate. Student's *t* test: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

shape. *Met*/*Gce* or *Kr-h1* was depleted by RNAi using *Lpp-Gal4*. The results revealed that the reduction of JH signaling in the adult fat body affected ovulation and egg shape, including reduced oviposition (Fig. 7*A*), increased ovary size (Fig. 7*B* and *SI Appendix*, Fig. S11 *A–A"*), accumulation of mature eggs (Fig. 7*C* and *SI Appendix*, Fig. S11 *B–B"*), enlarged abdomen (*SI Appendix*, Fig. S11*C*), muscle defects (Fig. 7 *D* and *E* and *SI Appendix*, Fig. S11 *D–D"* and Movie S17), reduced egg length (Fig. 7 *F* and *G* and *SI Appendix*, Fig. S11 *E–E"*), and reduced FC migration rate (Fig. 7*H* and *SI Appendix*, Fig. S11 *F–F"* and Movies S18–S20). Moreover, the depletion of JH-induced *collagen IV* expression using *Lpp-Gal4* phenocopied the above defects caused by reducing JH signaling in the adult fat body (Fig. 7*I–P* and *SI Appendix*, Fig. S12 *A–F"* and Movies S21–S24). It is notable that the reduction of neither JH signaling nor collagen IV expression in the adult fat body affected FC actin planar polarity (*SI Appendix*, Figs. S11 *G–G"* and S12 *G–G"*). Conclusively, JH-induced *collagen IV* expression in the adult fat body remotely contributes to ovarian muscle ECM assembly and function.

Discussion

JH Regulation of Female Reproduction in *Drosophila*. At the beginning of this study, we were very surprised to observe that, compared to the wild-type flies, the ovary size became smaller in the JH-deficient flies (30, 44), while it became larger with accumulated mature eggs in the mutants of JH receptors (Fig. 1). Nevertheless, in all these flies, the fecundity was significantly decreased with reduced oviposition (24, 30, 44, 47). Considering the previous work on JH-regulated reproduction in most insects (21, 22) and findings in this study, we conclude that JH signaling not only induces Vg production in the fat body but also promotes ovulation and maintains egg shape by affecting ovarian muscle contraction, while JH membrane signaling induces Vg uptake by initiating follicular patency in maturing oocytes in *Drosophila*. These actions of JH would explain the phenotypic difference between JH-deficient flies and JH receptor mutant flies. Notably, in the ovary, JH signaling and JH membrane signaling target ovarian muscles and FCs, respectively. Thus, the smaller ovary size in the JH-deficient flies is due to the lack of a majority of both JH signaling and JH membrane signaling (*SI Appendix*, Fig. S1), while the ovarian enlargement in Met^{27} or $Gce^{2.5K}$ is due to

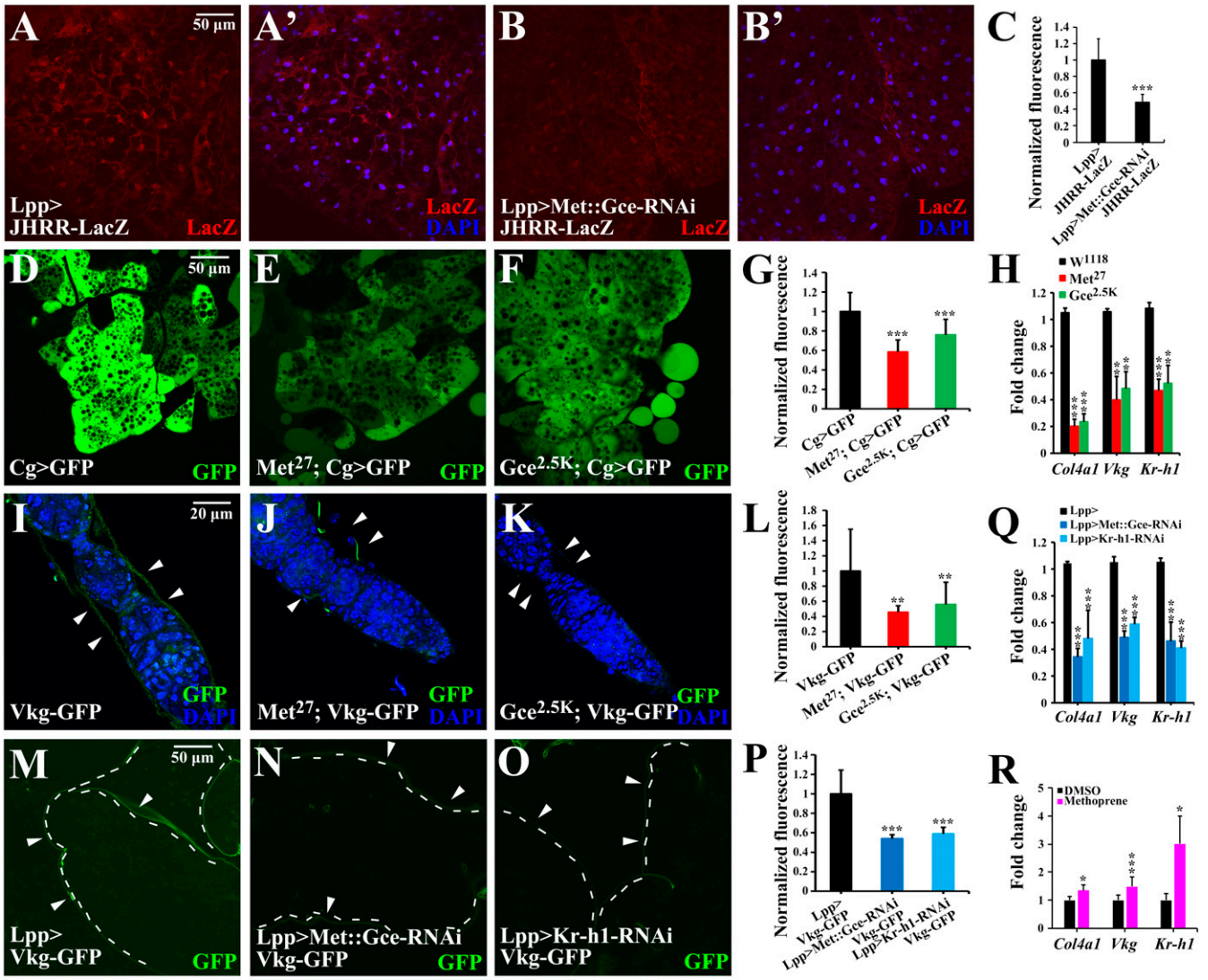

Fig. 6. JH signaling affects *collagen IV* gene expression in the adult fat body and deposition of collagen IV in ovarian muscles. (*A–C*) *JHRR-LacZ* expression is dramatically suppressed by down-regulation of *Me/Gce* using *Lpp-Gal4* compared with the control. LacZ is shown in red. Cell nuclei labeled with DAPI (blue). (*C*) Qualification of fluorescence intensity shown in *A–B′*. (*D–G*) The fluorescence intensity of *collagen IV* transcripts marker *Cg > GFP* in the adult fat body of *Met* or *Gce* mutants. (*G*) Quantification of fluorescence intensity shown in *D* and *E*. (*H*) qPCR analysis of *collagen IV* genes in the adult fat body of *Met* or *Gce* mutants. (*I–L*) The fluorescence intensity of *Vkg-GFP* in the ovarian muscle of *Met* or *Gce* mutants. The white arrowheads point to the epithelial sheath muscle. (*L*) Quantification of fluorescence intensity shown in *I–K*. (*M–P*) The fluorescence intensity of *Vkg-GFP* in the adult fat body of *Met/Gce* or *Kr-h1* RNAi flies using *Lpp-Gal4*. The white arrows point to adult fat body ECM and the adult fat body is outlined with white dotted line. (*P*) Quantification of fluorescence intensity shown in *M–O*. (*Q*) qPCR analysis of *collagen IV* genes in the adult fat body of *Met/Gce* or *Kr-h1* RNAi flies. (*R*) qPCR analysis of JH-regulated *collagen IV* genes in the *Jhamt*^{2} adult fat body treated with methoprene or DMSO control. Student's *t* test: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

the presence of JH membrane signaling and the partial lack of JH signaling (Fig. 1 and *SI Appendix*, Fig. S2).

In this study, we also compared the female reproductive defects in mated and unmated *Met*^{27} or *Gce*$^{2.5K}$ (Fig. 1 and *SI Appendix*, Fig. S2). Overall, the mated mutants exhibited similar but less significant phenotypic defects compared to the unmated mutants. Interestingly, JH could be transferred to the female from the males during copulation, and moreover, male seminal fluid proteins (i.e., sex peptide) stimulates JH biosynthesis of the females (52). The results suggest that mating-derived extra JH might partially override the phenotypic defects of *Met*^{27}or *Gce*$^{2.5K}$. In the future, it will be of great importance to identify the potential JH membrane receptor(s) and to better understand how JH signaling and JH membrane signaling coordinately regulate female reproduction in *Drosophila*.

JH Signaling Induces ECM Gene Expression for Ovarian Muscle ECM Assembly. Since ovulation deficiency and abnormal egg shape were observed in *Met*^{27} and *Gce*$^{2.5K}$ (Fig. 1), we investigated which type(s) of cells in the ovary respond to JH signaling. Surprisingly, JH signaling is mainly activated in the ovarian muscle cells (Fig. 2) and induces *laminin* expression in these cells (Fig. 5). Consistent with our previous study (34, 35), the adult fat body is also a major target tissue of JH signaling (Fig. 6 and *SI Appendix*, Fig. S10). JH signaling induces *collagen IV* expression in the adult fat body, from which collagen IV is secreted and deposited onto the ovarian muscles (Figs. 6 and 7, and *SI Appendix*, Fig. S12). This organ–organ cross talk is fascinating and merits future investigation. Since laminin locally and collagen IV remotely contribute to the ECM assembly of ovarian muscles, it is worthwhile to examine

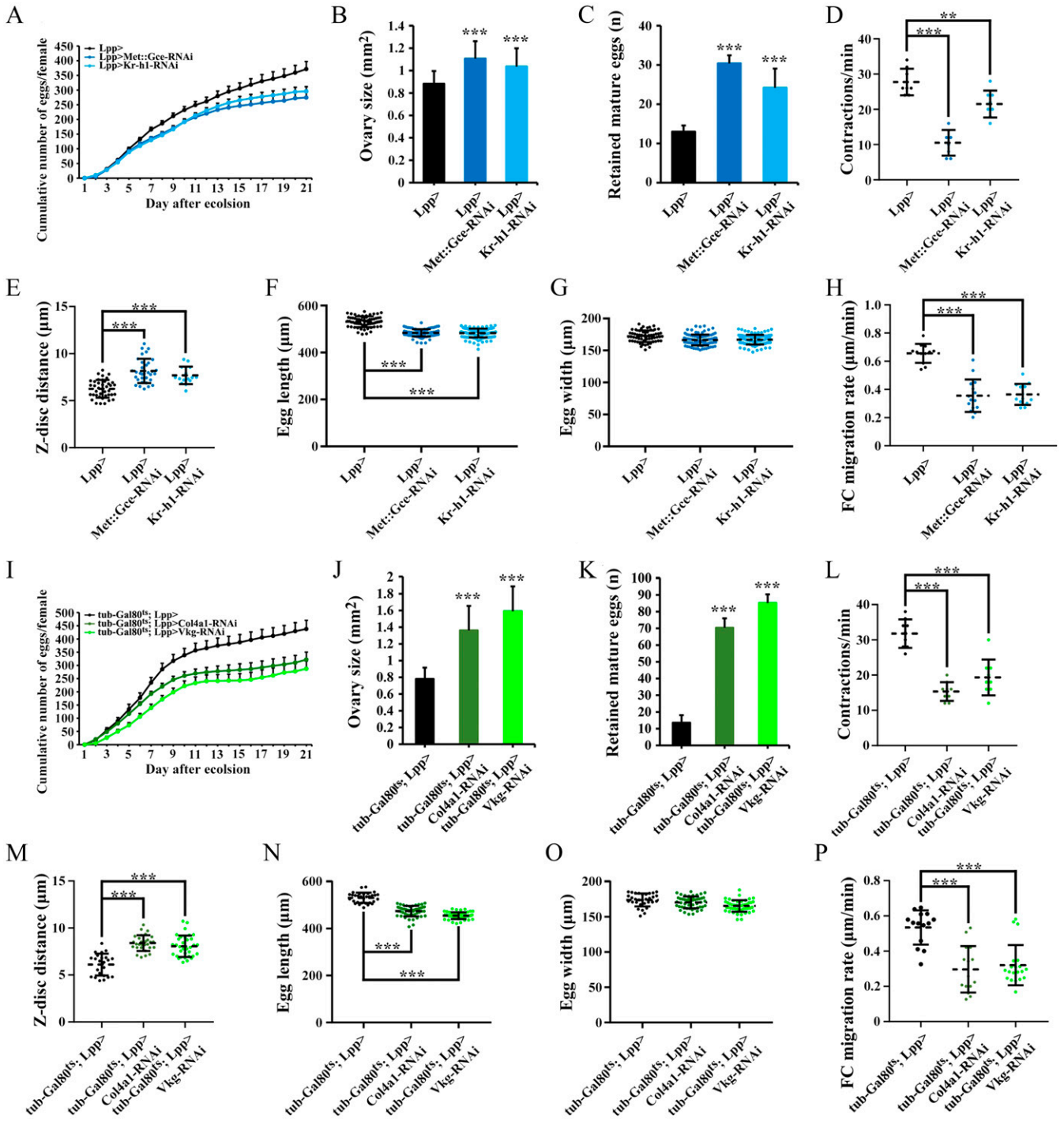

Fig. 7. Inhibition of JH signaling in the adult fat body down-regulates *collagen IV* gene expression, reduces ovulation, and disturbs egg shape. (*A–H*) RNAi depletion of *Met/Gce* or *Kr-h1* flies using *Lpp-Gal4* significantly reduced oviposition (*A*), increased ovarian size (*B*), accumulated mature eggs (*C*), weakened muscle contraction (*D*), elongated sarcomere (*E*), changed egg shape (*F* and *G*), and reduced FC migration rate (*H*). (*I–P*) RNAi depletion of *collagen IV* genes using *Lpp-Gal4* and *Tub-Gal80^{ts}* also significantly reduced oviposition (*I*), increased ovarian size (*J*), accumulated mature eggs (*K*), weakened muscle contraction (*L*), elongated sarcomere (*M*), changed egg shape (*N* and *O*), and reduced FC migration rate (*P*). Student's *t* test: $^{**}P < 0.01$ and $^{***}P < 0.001$.

whether and how JH signaling induces the expression of other ECM component genes (i.e., *nidogen* and *perlecan*) for the same physiology event. While Kr-h1 is a typical transcriptional repressor and inhibits the expression of 20E primary response genes during the larval stages (21), it becomes a critical question of how Kr-h1 mediates JH signaling to induce gene expression in adults.

ECM Components Are Indispensable for Ovarian Muscle Contraction and External Force Generation. The two types of ovarian visceral muscles are responsible for the slow, rhythmic involuntary contraction that mechanically generates an external power for the ovary or ovariole (15, 19). Each type of visceral muscle cells is covered by BM at both sides. Thus, the ECM components are indispensable for the ovarian muscle contraction to generate the

mechanical force, which functions to promote ovulation and maintain egg shape (Figs. 5 and 7). IRM genes, FGF, and Wnt signaling play roles for ovarian muscle contractions and the consequent power generation (16–18). It will be interesting to examine whether they are involved in the regulation of ovulation and egg shape. Moreover, it would be important to determine whether and how JH signaling interacts with these genes or signaling pathways. Because the ovarian muscle cells comprise a small portion of cells in the ovary, single-cell sequencing could be an ideal method to dissect the detailed molecular mechanism of how JH signaling helps the ovarian muscles to contract for power generation.

Ovulation is regulated by many physiological and neural signaling molecules, such as octopamine and 20E (11, 14). They both activate Mmp2, which is specifically expressed in the posterior FCs of stage 14 egg chambers and required for ovulation (11, 13, 14). In addition, ovulin, a male seminal protein, is transferred to the ovary during mating and thus activates octopamine signaling to induce ovulation (53). It is of great interest to examine whether and how these signaling molecules coordinately regulate ovulation.

Interestingly, the disorganization of ovarian muscle ECM assembly resembles the inhibition of JH signaling in ovarian muscles in the regulation of ovulation, but they are not identical in the regulation of egg shape. The inhibition of JH signaling in ovarian muscles affects egg chamber elongation and egg shape, partially through reducing FC collective migration and actin planar polarity (Fig. 4 and *SI Appendix*, Fig. S8). The disorganization of ovarian muscle ECM assembly also affects egg chamber elongation and egg shape. In these RNAi flies, FC collective migration is somehow reduced, while actin planar polarity is not changed (Figs. 5 and 7 and *SI Appendix*, Figs. S9 and S12). Therefore, it is likely that JH signaling regulates actin planar polarity independent of these ECM components. In addition, ovarian muscle contraction might affect egg shape by regulating Vg uptake by the maturing oocytes, while other unknown molecular mechanisms could be possible.

In conclusion, JH signaling promotes ovulation and maintains egg shape by inducing ECM gene expression (*SI Appendix*, Fig. S13). This important mechanism of JH signaling sheds light on hormone-regulated reproduction.

Materials and Methods

A detailed description of the materials and methods is given in *SI Appendix*, *SI Materials and Methods*. In brief, a number of fly strains and *Drosophila* genetics were used. Ovary size measurement and fecundity analysis, immunohistochemistry, live imaging of ovarian contractions, qPCR analysis, egg and sarcomere measurements, time-lapse image acquisition and microscopy, and JH analog treatment were performed. Reference *SI Appendix*, Table S1, for a list of primers used.

Data Availability. All study data are included in the article and/or supporting information.

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