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Antagonistic actions of juvenile hormone and 20-hydroxyecdysone within the ring gland determine developmental transitions in *Drosophila*

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In both vertebrates and insects, developmental transition from the juvenile stage to adulthood is regulated by steroid hormones. In insects, the steroid hormone, 20-hydroxyecdysone (20E), elicits metamorphosis, thus promoting this transition, while the sesquiterpenoid juvenile hormone (JH) antagonizes 20E signaling to prevent precocious metamorphosis during the larval stages. However, not much is known about the mechanisms involved in cross-talk between these two hormones. In this study, we discovered that in the ring gland (RG) of *Drosophila* larvae, JH and 20E control each other's biosynthesis. JH induces expression of a Krüppel-like transcription factor gene *Kr-h1* in the prothoracic gland (PG), a portion of the RG that produces the 20E precursor ecdysone. By reducing both steroidogenesis autoregulation and PG size, high levels of Kr-h1 in the PG inhibit ecdysteriod biosynthesis, thus maintaining juvenile status. JH biosynthesis is prevented by 20E in the corpus allatum, the other portion of the RG that produces JH, to ensure the occurrence of metamorphosis. Hence, antagonistic actions of JH and 20E within the RG determine developmental transitions in *Drosophila*. Our study proposes a mechanism of cross-talk between the two major hormones in the regulation of insect metamorphosis.

juvenile hormone | 20-hydroxyecdysone | ring gland | hormone biosynthesis | antagonistic action

The transition from the juvenile stage to adulthood is a key developmental event for reaching reproductive maturation. In animals, this process is regulated by steroid hormones and their corresponding nuclear receptors. In mammals, such regulatory steroids are two main classes of sex hormones: androgens in males and estrogens in females (1). In insects, major developmental transitions, including larval–larval molting and larval–pupal–adult metamorphosis, are elicited by pulses of 20-hydroxyecdysone (20E; the main active form of insect steroid hormones) (2). However, each major developmental transition in insects is coordinated by the sesquiterpenoid juvenile hormone (JH). The balance of the two hormones defines the outcome of each developmental transition: during the middle larval instars, a high level of JH ensures that 20E pulse only triggers larval–larval molting, while during the last larval instar, JH titer declines sharply and 20E pulse initiates metamorphosis (3–6). Thus, JH prevents 20E-induced metamorphosis and is therefore referred to as the "status quo" hormone (7, 8). A number of studies have shown either the effect of JH on 20E titer (9–12) or the effect of 20E on JH biosynthesis (13, 14), but the actual model of this mutual regulation has not been determined in any insect species (15, 16).

20E, in conjunction with its nuclear receptor complex composed of the ecdysone receptor (EcR) and ultraspiracle (USP) proteins, triggers a transcriptional cascade consisting of 20E primary-response genes and a subsequent array of 20E secondary-response genes, and thus induces each molt (2). During larval–pupal metamorphosis, 20E induces programmed cell death to eliminate larval tissues and promotes adult tissue formation from imaginal discs, mainly through two 20E primary-response genes, *Br-C* and *E93* (16, 17). Methoprene-tolerant (Met), a bHLH-PAS transcription factor, is a JH receptor that mediates the "status quo" actions of JH (18–20). *Krüppel homolog 1* (*Kr-h1*) is a JH primary-response gene that encodes a zinc-finger transcription factor (21–24). Kr-h1 transduces JH signals to prevent 20E-induced gene expression during larval stages to ensure that metamorphosis occurs only in the absence of JH and Kr-h1 (22, 25, 26). Therefore, Kr-h1 is regarded as an antimetamorphic factor (7, 8). The repression of *Br-C* and *E93* by Kr-h1 in peripheral target tissues partly accounts for the cross-talk between these two hormones (21–24, 27–31). Nevertheless, detailed studies are required to clarify the precise molecular mechanisms whereby Kr-h1 mediates the JH-20E cross-talk to achieve the antimetamorphic action at the level of hormone biosynthesis.

In the fruit fly, *Drosophila melanogaster*, a paralog of Met named Germ-cell expressed (Gce) functions as an alternate JH receptor (32–36). In the presence of JH, Met/Gce binds to a JH response region (JHRR; which contains three E-box–like motifs) in the *Kr-h1* promoter and activates *Kr-h1* expression (24). It is well documented that JH signaling is not essential during the early larval stages but is required for preventing metamorphosis (3, 4). The "status quo" action of JH in this dipteran insect is

Significance

In vertebrates, steroid hormones regulate developmental transition from juveniles to adults. Insect steroid hormone, 20-hydroxyecdysone (20E), coordinates with juvenile hormone (JH) to regulate metamorphosis; however, the precise cross-talk mechanism is not well understood. Here, we report that JH and 20E antagonize each other's biosynthesis in a major endocrine organ of *Drosophila* larvae: JH suppresses ecdysone biosynthesis and inhibits metamorphosis, whereas 20E suppresses JH biosynthesis and promotes metamorphosis. These data answer a long-standing question on how the mutual antagonism between the two major insect hormones regulates metamorphosis and may help to understand the hormonal regulation of developmental transition in mammals.

Author contributions: S. Liu and S. Li designed research; S. Liu, K.L., Y.G., X.L., W.C., and W.G. performed research; S. Liu, K.L., Y.G., X.L., W.C., W.G., Q.F., S.R.P., and S. Li analyzed data; and S. Liu, Q.F., S.R.P., and S. Li wrote the paper.

The authors declare no conflict of interest.

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comparatively subtle but crucial: JH prevents 20E-triggered programmed cell death of the larval fat body and differentiation of the optic lobe in the adult brain (3, 4, 33). Interestingly, the JH-deficient animals, *Met gce* double mutants, and *Kr-h1* mutants die around pupation with delayed developmental timing (the period from egg laying to pupariation) (3, 4, 11, 21, 33, 35, 36). These results are in contrast with those in the beetle, *Tribolium castaneum*, in which knockdown of *Met* or *Kr-h1* by systemic RNAi results in precocious metamorphosis (19, 22). It should be of great value to understand where and how JH signaling prevents 20E-induced metamorphosis by using the classic genetic and developmental model insect *Drosophila*.

Interestingly, we detected high levels of *Kr-h1* in the prothoracic gland (PG), a portion of the ring gland (RG), which produces the 20E precursor ecdysone. The RG is the major endocrine organ in *Drosophila* larvae, which consists of the PG producing ecdysone, the corpus allatum (CA) producing JH, and the corpus cardiacum producing a number of peptides including the adipokinetic hormone. We further determined that a major target organ of JH signaling is the PG, in which Kr-h1 transduces JH signals to inhibit ecdysone biosynthesis. Moreover, we discovered that JH and 20E, produced in the two different portions of the RG, inhibit each other's biosynthesis. This study answers a long-standing question on how the mutual antagonism between JH and 20E regulates insect metamorphosis.

Results and Discussion

***Drosophila* PG Shows High Levels of *Kr-h1* Expression When JH Titers Are High.** We have previously generated an LacZ reporter *JHRR-LacZ* based on the JHRR of *Drosophila Kr-h1* promoter, which recapitulates the responsiveness of *Kr-h1* to JH and Met/Gce (24). This reporter was employed to estimate the expression pattern of *Kr-h1* during the early wandering stage when the JH titers are high (ref. 24 and references therein). JHRR-LacZ was detected in various larval tissues, including the fat body, salivary glands, PG, and the adult midgut progenitor cells (AMP), but not in the larval midgut cells (Fig. S1, *Left*). PBac{Met-GFP. FPTB} is a transgenic line that carries a genomic BAC construct expressing the *Drosophila* Met protein that is C-terminally tagged with EGFP (Met-GFP), and this transgene is able to rescue the *Met gce* double mutants (Met^{27} $gce^{2.5K}$) to adults. Consistently, Met-GFP was also detected in the larval fat body, salivary glands, PG, and AMP, but not in the larval midgut cells (Fig. S1, *Right*). As revealed by a recent study using Bac recombineering and transgenic knock-in techniques, both *Met* and *gce* showed expression patterns similar to those shown in Fig. S1 (37). These results are consistent with the well-recognized role of JH signaling in targeting larval tissues to suppress the 20E-induced programmed cell death and in regulating the formation of adult organs during the larval–pupal metamorphosis (7, 8). Interestingly, we found that the PG shows abundant expression of *JHRR-LacZ* (Figs. S1, *Left* and S2 *A* and *A′*), but JHRR-LacZ was barely detected in the fat body and PG of the *Met gce* double mutants (24) (Fig. S2 *B* and *B′*). The expression of *JHRR-LacZ* in the PG was further confirmed by the colocalization of JHRR-LacZ with Spookier (Spok), a PG-specific enzyme catalyzing an essential step of ecdysone biosynthesis (38) (Fig. 1 *A* and *A‴*). Similarly, Met-GFP is highly expressed and colocalized with Spok in the PG (Fig. 1 *B* and *B‴*). Moreover, in situ hybridization revealed high *Kr-h1* expression in the PG of the wild-type animals but no detectable *Kr-h1* expression in the PG of the *Met gce* double mutants (Fig. 1 *C* and *D′* and Fig. S2 *C* and *C′*). Given that JH has been shown to regulate 20E titer in both *Drosophila* and the silkworm *Bombyx mori* (9–12), our observations imply that JH may control 20E titer by regulating ecdysone biosynthesis in the PG. These data suggest that JH exerts its action by modulating gene expression in multiple tissues, including the PG of *Drosophila*.

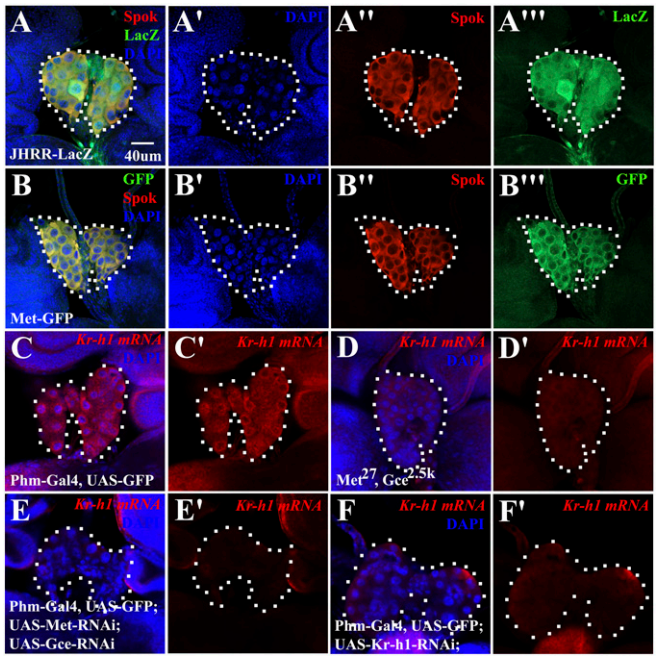

Fig. 1. Expression of JHRR-LacZ, Met-GFP, and *Kr-h1*. (*A–B‴*) Spok colocalizes with JHRR-LacZ and Met-GFP in the PG. (Scale bar, 40 μm.) (*A–A‴*) Spok (red), JHRR-LacZ (green), DAPI (blue). (*B–B‴*) Spok (red), Met-GFP (green), DAPI (blue). (*C–F′*) *Kr-h1* expression in the PG is significant in the wild-type larvae (*C* and *C′*) but decreased in the *Met gce* double mutant (*D* and *D′*), it is also decreased when *Met* and *Gce* or *Kr-h1* was knocked down by RNAi (*E–F′*). In situ hybridization was performed using antisense probes of *Kr-h1*. *Kr-h1* antisense (red), DAPI (blue).

Knockdown of *Met* and *gce* or *Kr-h1* in the PG Triggers Initiation of Metamorphosis. We then sought to investigate the possible roles of different target tissues in mediating JH actions. As reported previously (3, 4, 11, 21, 33, 35, 36), the JH-deficient animals *Aug21-Gal4*;*UAS-grim*, the double mutant Met^{27} $gce^{2.5K}$ animals, and the $Kr\text{-}h1^{K04411}$ mutants die around pupation with delayed rather than precocious developmental timing. Meanwhile, all of the three genotypes failed to undergo normal head eversion (Fig. 2 *A–C*). To investigate the function of JH signaling in different target tissues, we depleted expression of *Met* and *gce* or *Kr-h1* tissue-specifically using RNAi. Several Gal4 lines, including the PTTH-producing neuron-specific *PTTH-Gal4,* the fat body-specific *Lsp2-Gal4* and *Ppl-Gal4*, the AMP-specific *Esg-Gal4*, the salivary gland-specific *FKH-Gal4*, and the PG-specific *Phm-Gal4*, were individually crossed with *UAS-Met-RNAi*, *UAS-gce-RNAi*, or *UAS-Kr-h1-RNAi*. Knockdown of *Met* and *gce* or *Kr-h1* in PTTH-producing neurons, fat body, AMP, or salivary glands neither caused lethality nor significantly affected developmental timing (Fig. S3). In contrast, knockdown of *Met* and *gce* or of *Kr-h1* in the PG (Fig. 1 *E* and *F′*) resulted in complete lethality and failure of head eversion during the pupal stage, showing lethal phenotypes similar to *Aug21-Gal4*;*UAS-grim*, Met^{27} $gce^{2.5K}$, or $Kr\text{-}h1^{K04411}$ animals (Fig. 2 *D–F*). Consistent with our observations, a recent PG-specific RNAi screen study also showed that knockdown of *Kr-h1* in the PG resulted in pupal lethality (39). Moreover, knockdown of *Met* and *gce* or *Kr-h1* in the PG resulted in smaller body sizes and pupariation ∼36 h and 24 h earlier, respectively, compared with the pupariation time in wild-type animals (Fig. 2 *D–G*). Therefore, attenuation of JH signaling specifically in the PG accelerates larval development resulting in precocious metamorphosis, unlike in the JH-deficient animals, *Met gce* double mutants, and *Kr-h1* mutants. The tissue-specific RNAi results suggest that *Drosophila* PG is a key target organ mediating JH

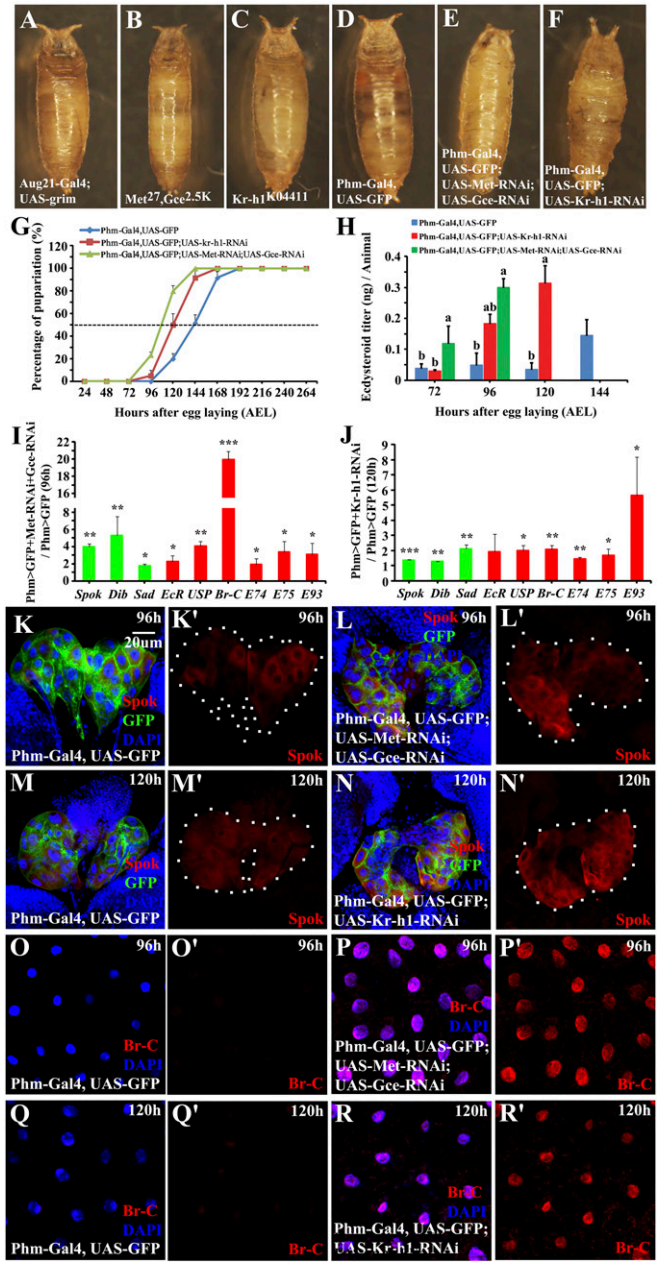

Fig. 2. Down-regulation of *Met* and *Gce* or *Kr-h1* in the PG results in an increase in ecdysone biosynthesis and precocious metamorphosis. (*A–F*) Lethal phenotypes; (*G*) developmental timing and percentage of pupariation; and (*H*) ecdysteroid titers of the indicated genotypes. (*I* and *J*) qRT-PCR measurements of gene expression. Fold changes shown are relative to control. Green bars indicate Halloween genes and red bars show key genes in the 20E-triggered transcriptional cascade. (*K–N′*) Spok protein level in the PG. Spok (red), GFP (green), DAPI (blue). (*O–R′*) Br-C protein level in the fat body. Br-C (red), DAPI (blue). For the *t* test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. ANOVA: bars labeled with different lowercase letters are significantly different ($P < 0.05$).

action, which is consistent with the high expression level of *Kr-h1* detected in the PG (Fig. S1).

JH Represses Ecdysone Biosynthesis in the PG. It is well documented that the timing of metamorphosis is coordinated by the rise in 20E titers (16). Therefore, we tested whether JH inhibits ecdysone biosynthesis in the PG and thus prevents premature pupariation (9–12). Indeed, knockdown of *Met* and *gce* or *Kr-h1* in the PG dramatically induced a premature increase in ecdysteroid (mainly 20E and ecdysone) titers (Fig. 2*H*). This premature increase in ecdysteroid titers is most likely due to an increase in ecdysone biosynthesis in the PG, as we detected an increase in mRNA levels of three Halloween genes (*Spok*, *Dib*, and *Sad*) (ref. 38 and references therein) as well as the protein level of Spok in response to the PG-specific knockdown of *Met* and *gce* or *Kr-h1* (Fig. 2 *I–N′*). Consistent with the increase in ecdysteroid titers, expression of *EcR*, *USP*, and several 20E primary-response genes (*Br-C*, *E74*, *E75*, and *E93*) in the whole body as well as the Br-C protein levels in the fat body were elevated in *Met gce* or *Kr-h1* RNAi animals (Fig. 2 *I*, *J*, and *O–R′*). These data show that JH normally represses ecdysone biosynthesis in the PG to prevent premature pupariation.

***Kr-h1* Overexpression in the PG Inhibits Ecdysone Biosynthesis and Blocks Metamorphosis.** To complement the loss-of-function studies, we tested whether PG-specific *Kr-h1* overexpression before the larval-pupal transition is sufficient to repress ecdysone biosynthesis and thus prevent metamorphosis. For this purpose, *Phm-Gal4* was combined with the temperature-sensitive Gal80 line *Tub-Gal80^{ts}* and then used to drive the expression of a *UAS-Kr-h1* transgene. Both *Phm-Gal4*;*Tub-Gal80^{ts}* and *Phm-Gal4*;*Tub-Gal80^{ts}*;*UAS-Kr-h1* larvae were first reared at a permissive temperature of 18°C until 120 h after egg laying (AEL) when they reached midthird instar, showing normal larval development. The larvae were then shifted to a restrictive temperature of 29°C. The control animals began to pupariate at 24 h after the shift, whereas the *Kr-h1*-overexpressing animals were arrested at the third instar, survived about 2 wk with overgrowth phenotypes, and never showed wandering behavior (Fig. 3 *A–C*). Following the addition of 20E to the diet at 144 h AEL, the *Kr-h1*–overexpressing animals initiated wandering behavior and pupariated within 24 h (Fig. 3*C*). At the restrictive temperature, ecdysteroid titers remained low and did not show an increase in the *Kr-h1*–overexpressing animals (Fig. 3*D*). At 168 h AEL, expression of *Spok*, *Dib*, and *Sad* was significantly inhibited by *Kr-h1* overexpression, and the Spok protein became undetectable in the PG (Fig. 3 *E–G′*). Moreover, expression of *EcR*, *USP*, and the other 20E-primary response genes tested decreased in the whole body of the *Kr-h1*–overexpressing animals (Fig. 3*E*), and the Br-C protein levels were undetectable in the fat body (Fig. 3 *H–I′*).

These loss-of-function and gain-of-function studies demonstrate that, through Kr-h1, JH prevents metamorphosis by inhibiting ecdysone biosynthesis in the PG. Previous studies identified PTTH and insulin-like peptides (ILPs) as positive regulators of ecdysone biosynthesis in *Drosophila* PG (6, 40, 41); our work establishes JH as a negative regulator of ecdysone biosynthesis.

JH Signaling in the PG Does Not Affect ILP Biosynthesis. In agreement with a previous study showing a premature increase in ecdysteroid titers in *Aug21-Gal4*;*UAS-grim* larvae (11), we also observed an increase in ecdysteroid titers in both *Met^{27} gce$^{2.5K}$* and *Kr-h1^{K04411}* larvae (Fig. S4*A*). Thus, either the complete absence of JH signaling or the depletion of JH signaling in the PG causes a premature increase in ecdysteroid titers. However, their opposite effects on developmental timing were not fully understood. A number of studies have demonstrated that developmental timing depends on 20E and insulin/ ILP signals, which mainly control growth period and growth rate, respectively. Moreover, triangular interplays might exist among 20E, ILPs, and JH in insects. For example, ILPs promote ecdysone biosynthesis, and 20E inhibits ILP biosynthesis, forming a negative feedback loop (42–44). JH and 20E mutually affect each other's biosynthesis (9–14). JH and ILPs mutually promote each other's biosynthesis, forming a positive feedback loop (11, 45–47). Therefore, we compared whether the depletion of JH signaling in the PG and the absence of JH signaling in the whole animals differently affect ILP biosynthesis and thus insulin/ILP signaling [IIS; high expression of

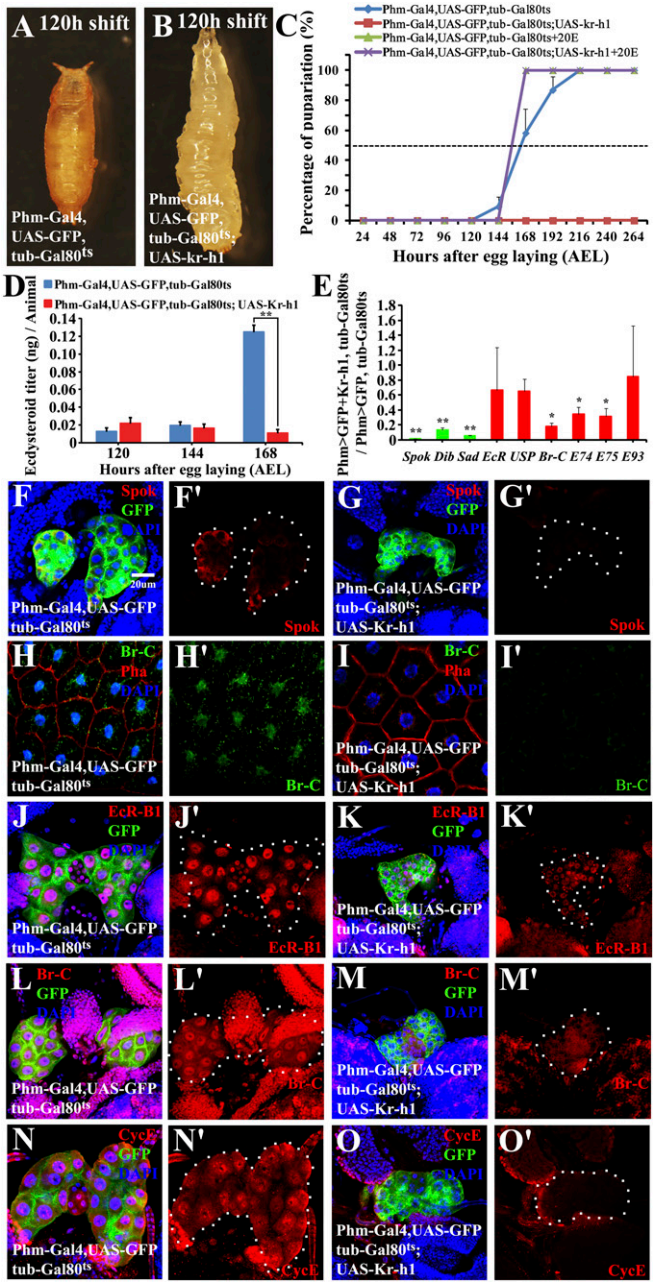

Fig. 3. By reducing both steroidogenesis autoregulation and PG size, overexpression of *Kr-h1* in the PG decreases and delays ecdysone biosynthesis and prevents metamorphosis. (*A* and *B*) Developmental arrest in larvae with PG-specific *Kr-h1* overexpression. (*C*) Developmental timing and percentage of pupariation. Added 20E at 144 h AEL. (*D*) Ecdysteroid titers. (*E*) qRT-PCR measurement of gene expression. Fold changes are relative to control. (*F*–*G*′) Spok protein level in the PG. Spok (red), GFP (Green), DAPI (blue). (*H*–*I*′) Br-C protein level in the fat body. Br-C (green), phalloidin (red), DAPI (blue). (*J*–*O*′) EcR-B1, Br-C, and CycE levels in the PG. EcR-B1, Br-C, and CycE (red), GFP (green), DAPI (blue). For the *t* test: $*P < 0.05$; $**P < 0.01$.

InR and *4EBP* represents low IIS, and vice versa (11)] during the feeding stages of the third larval instar. Importantly, expression of *ILP1-7*, *4E-BP*, and *InR* was not altered when *Met* and *gce* or *Kr-h1* was knocked down in the PG (Fig. S4 *B* and *C*). Thus, when JH signaling is only abolished in the PG, ecdysone biosynthesis is enhanced, while ILP biosynthesis and IIS are not affected, resulting in precocious metamorphosis. A previous study has shown increased expression of *InR* and *4EBP* and thus decreased IIS in *Aug21-Gal4;UAS-grim* during the feeding stages of the third larval instar (11). Importantly, similar observations were detected in Met^{27} $gce^{2.5K}$ (Fig. S4*D*). However, expression of ILPs either increased or decreased in this mutant (Fig. S4*D*). These results show that in the complete absence of JH signaling, ecdysone biosynthesis is enhanced, but IIS is reduced, thus developmental timing is delayed. Besides the PG, there should be another target tissue that mediates JH signaling to regulate growth rate and developmental timing. The data also imply that besides altering expression of ILPs, JH might alter IIS through regulating other physiological processes, such as feeding behavior and nutrient status.

JH Signaling Suppresses Ecdysone Biosynthesis by Reducing Both Steroidogenesis Autoregulation and PG Size. We next pursued the mechanisms by which JH signaling inhibits ecdysone biosynthesis. It is well documented that a fine regulatory loop exists between ecdysone biosynthesis and 20E signaling in *Drosophila* and *Bombyx* (48–52). At least EcR, Br-C, and E75 are involved in the feedback regulation of Halloween gene expression and thus ecdysone biosynthesis in *Drosophila* PG (48–50). The feedback regulation of ecdysone biosynthesis by 20E signaling is often referred to as steroidogenesis autoregulation, which plays a key role in 20E signaling during metamorphosis (16). We thus wondered whether JH modulates ecdysone biosynthesis at the level of steroidogenesis autoregulation. Indeed, up-regulation of JH signaling by *Kr-h1* overexpression resulted in a significant decrease in the protein levels of EcR-B1 and Br-C in the PG (Fig. 3 *J*–*M*′). Conversely, knockdown of *Met* and *gce* or *Kr-h1* in the PG led to an increase in the protein levels of EcR-B1 and Br-C in the PG (Fig. S5). In *Drosophila* Kc cells, *Kr-h1* overexpression directly inhibits expression of *EcR*, *Br-C*, and *E75*, irrespective of whether 20E is absent or present in the medium (Fig. S6). Therefore, through Kr-h1, JH signaling inhibits ecdysone biosynthesis in *Drosophila* PG by reducing the positive feedback.

Previous studies have shown that the normal PG size is critical for ecdysone biosynthesis (43, 44, 53). The PG size was significantly reduced by *Kr-h1* overexpression (Fig. 3 *F*, *G*′, and *J*–*O*′), accompanied by reduced CycE protein level and cell size (Fig. 3 *N*–*O*′ and Fig. S7 *A*–*C*). Interestingly, *EcR* RNAi in the PG did not affect the organ size, CycE protein level, and cell size, but knockdown of *EcR* in the PG that overexpressed *Kr-h1* reduced all three parameters similarly to those in the PG that overexpressed *Kr-h1* alone (Fig. S8). Nevertheless, PG size was normal in *Aug21-Gal4;UAS-grim*, Met^{27} $gce^{2.5K}$, and $Kr\text{-}h1^{K04411}$ animals (3, 4, 11, 21, 33, 35, 36). Consistently, PG size was not affected by PG-specific knockdown of *Met* and *gce* or *Kr-h1* (Figs. S5 and S7*D*). These results suggest that high JH signaling is able to reduce PG size in a Kr-h1–dependent but 20E-independent manner. The detailed molecular mechanisms of Kr-h1–mediated reduction in PG size warrant further investigation, although CycE protein level is involved in regulating the endocycling of PG cells (53). Hence, JH signaling reduces steroidogenesis autoregulation by suppressing 20E signaling in the PG, while JH signaling reduces PG size independently of 20E signaling.

Taken together, our results show that one of the major target organs of JH signaling is the PG, in which Kr-h1 mediates JH signaling to inhibit ecdysone biosynthesis through a reduction in both steroidogenesis autoregulation and PG size.

20E Prevents JH Biosynthesis in the CA to Permit Metamorphosis. Having demonstrated that JH inhibits ecdysone biosynthesis in the PG, we examined whether vice versa, 20E also targets the CA to regulate JH biosynthesis in *Drosophila*, as observed in other insects (13, 14). *Jhamt-Gal4*, which has a more robust CA-specific expression than *Aug21-GAL4* (33, 36), was crossed with *UAS-EcR-RNAi* to specifically reduce *EcR* expression in the CA by RNAi (Fig. S9 *C* and *D*′). Surprisingly, knockdown of *EcR* in the CA resulted in complete lethality during the pupal stage (Fig.

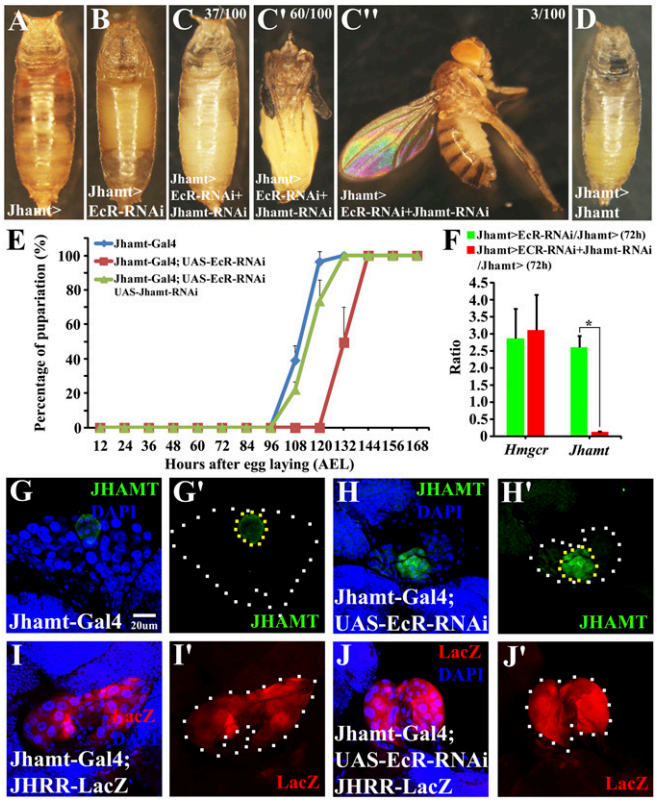

Fig. 4. Down-regulation of *EcR* in the CA increases JH biosynthesis, which decreases and delays ecdysone biosynthesis and prevents metamorphosis. (*A*–*C*″) CA-specific *EcR* depletion resulted in complete animal lethality at the pupal stage, which was partially rescued by concurrent *Jhamt* depletion. Note: 60 out of 100 animals were rescued to the pharate adult stage (*C*′) and three to the adult stage (*C*″). (*D*) CA-specific *Jhamt* overexpression resulted in complete animal lethality at the pupal stage. (*E*) Developmental timing and percentage of pupariation. (*F*) qRT-PCR measurements of gene expression in the brain-RG complex. Fold changes are relative to control. (*G*–*H*′) JHAMT protein level in the CA. JHAMT (green), DAPI (blue). (*I*–*J*′) *JHRR-LacZ* (*Kr-h1*) expression in the PG. JHRR-LacZ (red), DAPI (blue). For the *t* test: $*P < 0.05$.

4 *A* and *B*); it also delayed pupariation by ∼24 h (Fig. 4*E*). At 72 h AEL, knockdown of *EcR* in the CA induced a two- to threefold increase in the mRNA levels of the two key enzymes (JHAMT and HMGCR) of JH biosynthesis (Fig. 4*F*). At 96 h AEL, CA-specific knockdown of *EcR* significantly increased the JHAMT protein level in the CA and *JHRR-LacZ* expression in the PG (Fig. 4 *G*–*J*′), demonstrating elevated JH biosynthesis and JH signaling. Mechanistically, 20E signaling inhibition in the CA attenuates and delays ecdysone biosynthesis in the PG by reducing steroidogenesis autoregulation and PG size (Figs. S7 *E* and *F* and S9). Showing elevated JH biosynthesis and JH signaling, knockdown of *EcR* in the CA led to phenotypic changes similar to but much weaker than those caused by *Kr-h1* overexpression in the PG for a period of 48 h during the third larval instar (Fig. 3). Importantly, the lethality caused by knockdown of *EcR* in the CA was partially rescued by the simultaneous knockdown of *Jhamt* in the CA (Fig. 4 *C* and *C*″), which strongly supports the conclusion that elevated JH prevents normal development upon knockdown of *EcR* in the CA. Consistently, low ecdysteroid titers and 20E signaling caused by CA-specific *EcR* RNAi were rescued by concurrent *Jhamt* RNAi (Fig. S10). These results suggest that blocking 20E signaling in the CA results in elevated JH biosynthesis, which, in turn, leads to enhanced JH signaling in the PG, thus preventing ecdysone biosynthesis and 20E-induced metamorphosis. This study demonstrates 20E as an extracellular signal to regulate JH biosynthesis in *Drosophila*.

The previous *Drosophila* white pupal bioassay has shown that topical application of JH analogs on the white prepupae causes pupal lethality (ref. 35 and references therein). To confirm if elevated JH biosynthesis was sufficient to block 20E-induced metamorphosis, *Jhamt-Gal4* was crossed with *UAS-Jhamt. Jhamt* overexpression in the CA to increase JH biosynthesis (54) and JH signaling caused complete lethality during the pupal stage (Fig. 4*D* and Fig. S11). These data suggest that *Drosophila* employs 20E signaling to prevent JH biosynthesis in the CA so that the PG produces sufficient ecdysone to ensure the occurrence of metamorphosis. Together, these results purport JH and 20E to be the key factors involved in the reciprocal regulation of ecdysone and JH biosynthesis, respectively, in the RG, thus providing a model of cross-talk between these two hormones in the regulation of insect metamorphosis (Fig. 5).

In summary, JH signaling through Kr-h1 inhibits ecdysone biosynthesis in the PG to prevent metamorphosis, and vice versa, 20E prevents JH biosynthesis in the CA to ensure the occurrence of metamorphosis. JH and 20E, the two most important insect hormones regulating major developmental transitions, mutually inhibit the biosynthesis of each other, thus forming a regulatory network controlling metamorphosis (Fig. 5). Hence, antagonistic hormone actions within the RG determine developmental transitions in *Drosophila*. Since Krüppel-like factors are essential effectors of nuclear receptor signaling (55), it will be of great interest to examine whether Krüppel-like factors also mediate sex steroid signals to regulate developmental transitions from the juvenile stage to adulthood in mammals.

Materials and Methods

A detailed description of the materials and methods used in this study is provided in *SI Materials and Methods*. A number of fly strains and *Drosophila* genetics were used. Immunostaining, in situ hybridization, and

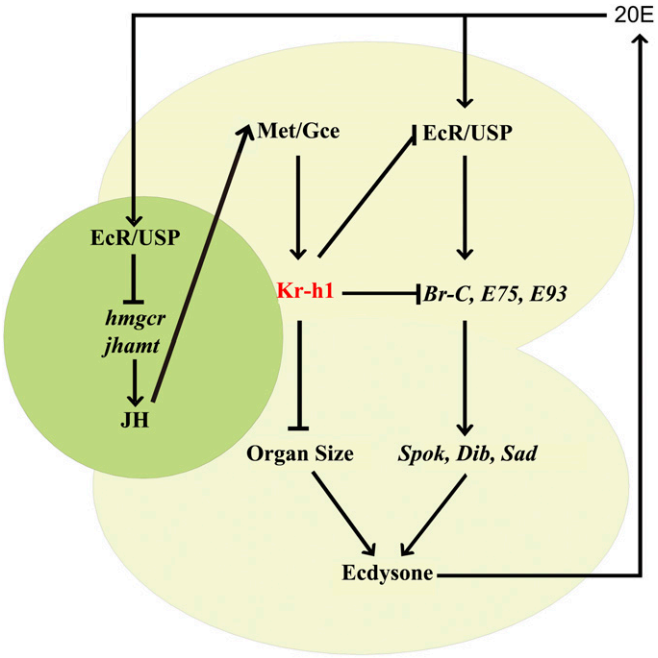

Fig. 5. A regulatory network of biosynthesis and action of 20E and JH in the RG. Green: CA producing JH; yellow: PG producing ecdysone; CA and PG are two portions of the RG. JH signaling through Kr-h1 inhibits ecdysone biosynthesis in the PG to prevent metamorphosis, while 20E signaling prevents JH biosynthesis in the CA to permit metamorphosis. Thus, antagonistic hormone actions within the RG determine developmental transitions in *Drosophila*.

imaging were performed. Developmental timing was analyzed by recording pupariation. Measuring ecdysteroid titers, 20E feeding experiments, cell culture, and qPCR were previously described. See Table S1 for a list of all primers used.

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