Krüppel homolog 1 represses insect ecdysone biosynthesis by directly inhibiting the transcription of steroidogenic enzymes

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In insects, juvenile hormone (JH) and the steroid hormone ecdysone have opposing effects on regulation of the larval–pupal transition. Although increasing evidence suggests that JH represses ecdysone biosynthesis during larval development, the mechanism underlying this repression is not well understood. Here, we demonstrate that the expression of the Krüppel homolog 1 (Kr-h1), a gene encoding a transcription factor that mediates JH signaling, in ecdysone-producing organ prothoracic gland (PG) represses ecdysone biosynthesis by directly inhibiting the transcription of steroidogenic enzymes in both *Drosophila* and *Bombyx*. Application of a JH mimic on ex vivo cultured PGs from *Drosophila* and *Bombyx* larvae induces *Kr-h1* expression and inhibits the transcription of steroidogenic enzymes. In addition, PG-specific knockdown of *Drosophila Kr-h1* promotes—while overexpression hampers—ecdysone production and pupariation. We further find that Kr-h1 inhibits the transcription of steroidogenic enzymes by directly binding to their promoters to induce promoter DNA methylation. Finally, we show that Kr-h1 does not affect DNA replication in *Drosophila* PG cells and that the reduction of PG size mediated by *Kr-h1* overexpression can be rescued by feeding ecdysone. Taken together, our data indicate direct and conserved Kr-h1 repression of insect ecdysone biosynthesis in response to JH stimulation, providing insights into mechanisms underlying the antagonistic roles of JH and ecdysone.

juvenile hormone | Kr-h1 | ecdysone biosynthesis | transcriptional repression | direct regulation

Endocrine regulation of the developmental transition of animals from an immature juvenile to a reproductive adult stage is a fundamental problem in developmental biology and physiology. In insects, two major endocrine hormones, the steroid hormone ecdysone, which is derived from the prothoracic gland (PG), and juvenile hormone (JH), which is derived from the corpora allata (CA), coordinately control developmental transitions in opposite manners (1, 2). In *Drosophila*, for example, ecdysone pulses appear at later stages of each larval instar and trigger larval molting or larval–pupal metamorphosis via a precise signaling cascade (1, 3, 4), while high JH titer is present at early stages of each larval instar and is coupled with insulin signaling to maintain growth (2, 3, 5). In addition, *Drosophila* JH has been shown to repress ecdysone biosynthesis and signaling to prevent larvae from precociously turning into adults. CA ablation, or double mutants of the two JH receptors, *Methoprene tolerant* (*Met*) and *germ-cell expressed* (*gce*), promote ecdysone biosynthesis and up-regulate the expression of the *Broad complex* (*BR-C*) involved in ecdysone signaling, which in turn induces precocious ecdysone action (5–7).

The antagonistic effects of JH on ecdysone action have also been analyzed in other insect species. Fluctuation of JH and ecdysone titers during *Bombyx* larval development is also opposite (8, 9). JH was first shown in *Bombyx* to indirectly modulate the release of ecdysone by inhibiting PG responsiveness to brain-produced prothoracicotropic hormone peptide (10). In contrast, deficiency of either JH biosynthesis or JH signaling results in precocious metamorphosis; such a deficiency can be induced by overexpression of *juvenile hormone esterase*, mutation in the cytochrome P450 gene *CYP15C1*, or mutation of JH receptors (11–13).

The transcription factor Krüppel homolog 1 (Kr-h1) has been identified as a key regulator that mediates the repressive action of JH on insect metamorphosis (2, 14). JH acts together with the Met/Gce receptors to directly induce *Kr-h1* expression (15–17). Importantly, reduction of *Kr-h1* activity in the whole body causes precocious metamorphosis (15, 18, 19), whereas overexpression of *Kr-h1* in the *Drosophila* epidermis or *Bombyx* whole body disrupts the larval–pupal transition (16, 20). In addition, Kr-h1 was also found to negatively regulate ecdysone action by decreasing the expression of ecdysone signaling genes *BR-C* and *E93* in response to JH (21, 22). Although these findings partially reveal that Kr-h1 can mediate the antagonizing regulation of JH on ecdysone action, the mechanism underlying systemic regulation other than ecdysone signal transduction remains largely unknown.

In this study, we demonstrate a direct regulation of Kr-h1–mediated JH signaling in inhibiting ecdysone biosynthesis in the PGs of *Drosophila* and *Bombyx*. We find that JH mimic (JHM)

Significance

Two major endocrine hormones in insects, juvenile hormone (JH) and ecdysone, regulate developmental transitions and growth duration in an opposite manner. Deciphering the mechanisms underlying cross-talk between JH and ecdysone is key to understanding the control of insect growth and development. We show that in both *Drosophila* and *Bombyx*, JH directly acts on the prothoracic gland to induce *Krüppel homolog 1* (*Kr-h1*) expression, which in turn represses ecdysone biosynthesis by reducing the transcription of steroidogenic enzymes. This transcriptional repression is mediated by direct binding of Kr-h1 to target gene-promoter regions, inducing epigenetic promoter DNA methylation. Our findings provide insights into the direct repression of ecdysone biosynthesis by Kr-h1–mediated JH signaling in insect prothoracic gland.

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application on ex vivo-cultured *Drosophila* brain–ring gland (brain–RG) complex containing the PG or *Bombyx* PG elevates *Kr-h1* expression but inhibits the transcription of steroidogenic enzymes. PG-specific knockdown or overexpression analysis revealed that *Kr-h1* expression in *Drosophila* PG suppresses developmental transition by reducing both transcription of steroidogenic enzymes and ecdysone production. Mechanistically, Kr-h1 suppresses steroidogenic enzyme transcription by directly binding to potential Kr-h1 binding site (KBS) in the promoters of steroidogenic enzyme genes and inducing promoter DNA methylation. Taken together, our results identify a conserved mechanism by which Kr-h1 negatively regulates ecdysone biosynthesis by directly inhibiting transcription of steroidogenic enzymes in *Drosophila* and *Bombyx*.

Results

JHM Delays Pupariation and Inhibits Ecdysone Biosynthesis and Transcription of Steroidogenic Enzymes in *Drosophila* and *Bombyx*. Previous studies have shown that JH cooperates with ecdysone to regulate insect growth and development (3). To explore the mechanism underlying JH regulation of ecdysone biosynthesis, we treated *Drosophila* and *Bombyx* larvae with the JHM methoprene. Interestingly, continuous feeding of JHM starting at 48 h after egg laying (AEL) resulted in a pupariation delay of 12∼16 h in *Drosophila* (Fig. 1*A*). Similarly, application of JHM to the body of *Bombyx* larvae on the second day of the last larval instar delayed larval–pupal transition, namely JHM-treated *Bombyx* larvae still kept feeding while control larvae had stopped feeding and entered the wandering stage (Fig. 1*A′*). Given that ecdysone controls insect developmental transitions (1), we further checked the effect of JHM treatment on ecdysone production by measuring ecdysteroid titer via enzyme immunoassay (EIA). As expected, JHM treatment decreased ecdysone production in both *Drosophila* and *Bombyx* (Fig. 1 *B* and *B′*).

Insect ecdysone is synthesized in the PG during larval development and is mediated by several well-studied steroidogenic enzymes, including Nvd, Sro, Spok/Spo, Cyp6t3, Phm, Dib, and Sad (4, 23–25). Therefore, we tested whether JH affects transcription of steroidogenic enzymes. We first used JHM methoprene to treat ex vivo-cultured brain–RG complex containing PG from *Drosophila* at the beginning of puparation, a stage when the ecdysone titer is relatively high. Subsequent qRT-PCR analysis demonstrated that transcription of most steroidogenic enzymes was decreased in *Drosophila* PGs following JHM application (Fig. 1*C*). Similarly, with JHM treatment on ex vivo-cultured PGs from *Bombyx* at the beginning of larval–pupal transition, all steroidogenic enzyme genes were also transcriptionally reduced (Fig. 1*C′*). These data revealed that JH can directly act on the PG to inhibit transcription of steroidogenic enzymes.

Expression Levels of the JH Signal Transducer Kr-h1 in *Drosophila* PG Affect Larval Development, Ecdysone Biosynthesis, and Transcription of Steroidogenic Enzymes. Previous reports have revealed that the transcription factor Kr-h1 mediates JH signaling and is positively transcriptionally regulated by the JH receptor complex that includes Met and Gce (2, 6, 14, 17). Our data revealed that Kr-h1 expression in the PGs of both *Drosophila* and *Bombyx* larvae is relatively high at the early stage of each larval instar and declines before developmental transitions (Fig. S1 *A–C*), which is consistent with changes in JH titer observed during larval development (3, 8). JHM application on ex vivo-cultured PGs from either *Drosophila* or *Bombyx* larvae significantly increased *Kr-h1* transcription (Fig. S2 *A* and *B*). Interestingly, we observed that the *Drosophila* steroidogenic enzyme Spok, a rate-limiting enzyme involved in insect ecdysone biosynthesis (26), was expressed at low

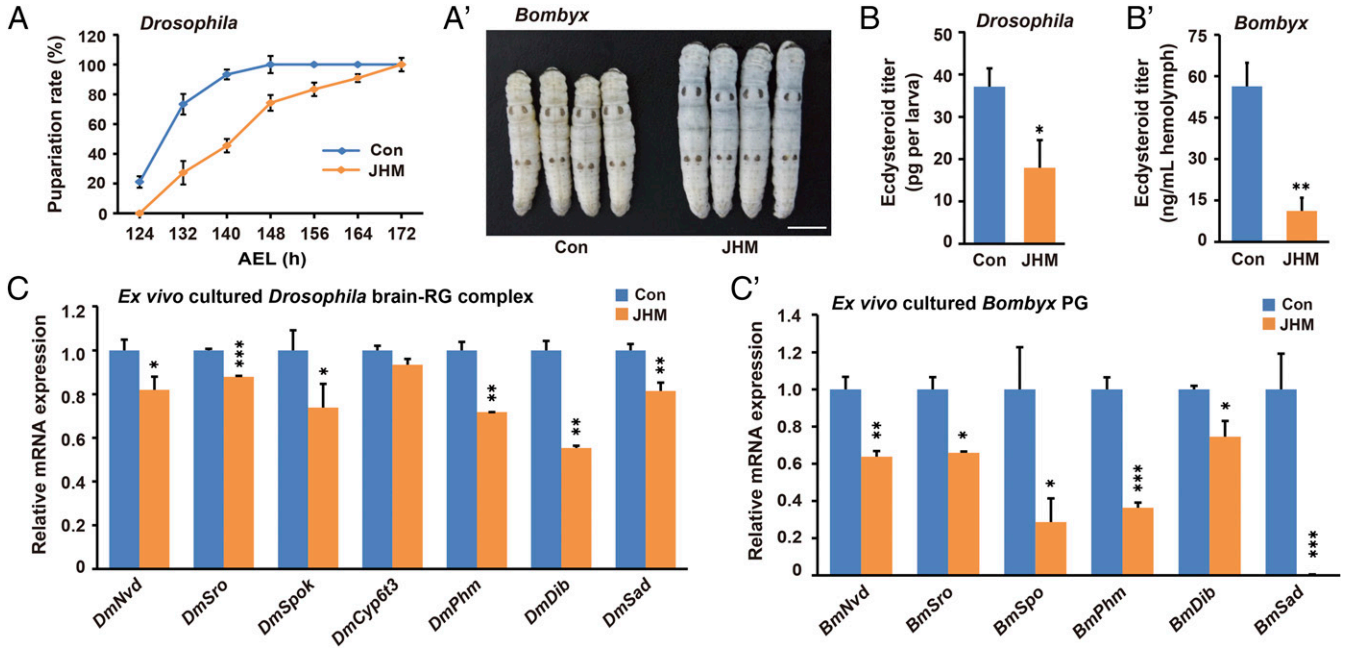

Fig. 1. JHM application delays pupariation and inhibits both ecdysone biosynthesis and transcription of steroidogenic enzyme genes in *Drosophila* and *Bombyx*. (*A* and *A′*) The application of JHM methoprene on both *Drosophila* and *Bombyx* larvae results in developmental delay. *Drosophila* wild-type *yw* larvae at 24 h AEL were fed on food supplemented with either ethanol as control (con) or JHM (*A*). JHM application on the body of *Bombyx* larvae was performed on the second day of the last larval instar (*A′*). (Scale bar, 1 cm.) (*B* and *B′*) Ecdysone titer changes in the whole body of *Drosophila* larvae and in the hemolymph of *Bombyx* larvae after JHM treatment. Animals treated with ethanol in *Drosophila* or acetone in *Bombyx* were used as controls. (*C* and *C′*) qRT-PCR analysis of the changes in steroidogenic enzyme mRNA levels in ex vivo cultured *Drosophila* brain–RG complex and *Bombyx* PG following JHM application. *Drosophila* brain–RG complex and *Bombyx* PG were isolated from animals at the beginning of larval–pupal transition. All experiments were performed in three biological replicates. Values are represented as the mean ± SE (error bars). For the significance test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control.

level 84 h AEL but was highly expressed at 120 h AEL (Fig. S1*D*), revealing an opposite pattern of *Spok* expression and ecdysone production with Kr-h1 expression.

To elucidate whether the expression level of the JH signal transducer *Kr-h1* in the PG can affect ecdysone biosynthesis, we knocked down *DmKr-h1* by RNAi using the PG-specific driver Phm-Gal4. PG-specific knockdown of *DmKr-h1* resulted in precocious pupariation by about 16 h (Fig. 2*A*), increased ecdysone production (Fig. 2*B*), and elevated expression of the ecdysone primary response gene *DmE75B* in the fat body (Fig. 2*B*). Expression of steroidogenic enzyme genes, such as *Nvd*, *Sro*, *Spok*, *Cyp6t3*, *Phm*, and *Sad*, was also up-regulated following *DmKr-h1* knockdown (Fig. 2*C* and Fig. S3*A*). Similarly, reduction in *DmKr-h1* levels in heterozygotes ($DmKr\text{-}h1^{+/-}$) of an enhancer-trap line of $DmKr\text{-}h1^{10642}$, also caused precocious pupariation, increases in ecdysone production, and fat-body expression of the *DmE75B* gene, and an up-regulation of steroidogenic enzyme gene expression (Fig. S4).

We further investigated the effect of PG-specific *DmKr-h1* overexpression driven by Phm-Gal4 on *Drosophila* ecdysone biosynthesis. *DmKr-h1* overexpression in the PG caused developmental arrest at the L1 stage (Fig. 2*D*), similar to the phenotype observed for PG-specific knockdown of *Drosophila* steroidogenic enzymes (27). Compared with the control, both ecdysone biosynthesis and fat-body expression of *DmE75B* were significantly suppressed by PG-specific *DmKr-h1* overexpression (Fig. 2*E*). *DmKr-h1* overexpression in the PG also led to decreased transcription of steroidogenic enzyme genes, including *Nvd*, *Spok*, *Phm*, *Dib*, and *Sad* and *Spok* expression was undetectable at 48 h AEL (Fig. 2*F* and Fig. S3*B*). Strikingly, the L1 arrest caused by PG-specific *DmKr-h1* overexpression could be rescued by feeding ecdysone or 20-hydroxyecdysone (20E) as an active derivative of ecdysone (Fig. 2*G*), and ~50% of L1-arrested larvae could develop to pupariation (Fig. S5). These results, together with the effects of both JHM application on the PGs and *DmKr-h1* knockdown in the PG, indicate that Kr-h1–mediated JH signaling in insect PGs represses ecdysone biosynthesis by inhibiting transcription of steroidogenic enzymes.

Kr-h1 Negatively Regulates Steroidogenic Enzyme Gene-Promoter Activity. Increasing evidence has demonstrated that the transcription of steroidogenic enzymes is directly regulated by multiple transcription factors (24). Recent reports in *Bombyx* also confirmed that Kr-h1 can bind to a specific *cis*-regulatory element KBS on the promoters of genes involved in ecdysone signaling, namely *BR-C* and *E93*, to negatively regulate their transcription (21, 22). Accordingly, we investigated the possibility that Kr-h1 may repress transcription of steroidogenic enzymes by directly binding to the potential KBSs in their promoters. First, our prediction analysis showed that the steroidogenic enzyme genes, *Nvd*, *Sro*, *Spok*/*Spo*, *Cyp6t3*, *Phm*, *Dib*, and *Sad*, from *Drosophila* and *Bombyx*, contain potential KBSs within the ~3-kb sequence upstream of their translational start sites (Fig. S6). To examine Kr-h1 regulation of steroidogenic enzyme gene-promoter activity, we cloned these upstream sequences into a luciferase-reporter vector and transfected them into *Drosophila* S2 and *Bombyx* BmE cells, followed by either JHM treatment or *Kr-h1* overexpression. Subsequent dual-luciferase reporter assays revealed that the activities of most of the promoters for steroidogenic enzyme genes from both *Drosophila* and *Bombyx* were significantly decreased after either JHM application or *Kr-h1* overexpression (Fig. 3 *A*–*B*′ and Fig. S2 *C*–*F*).

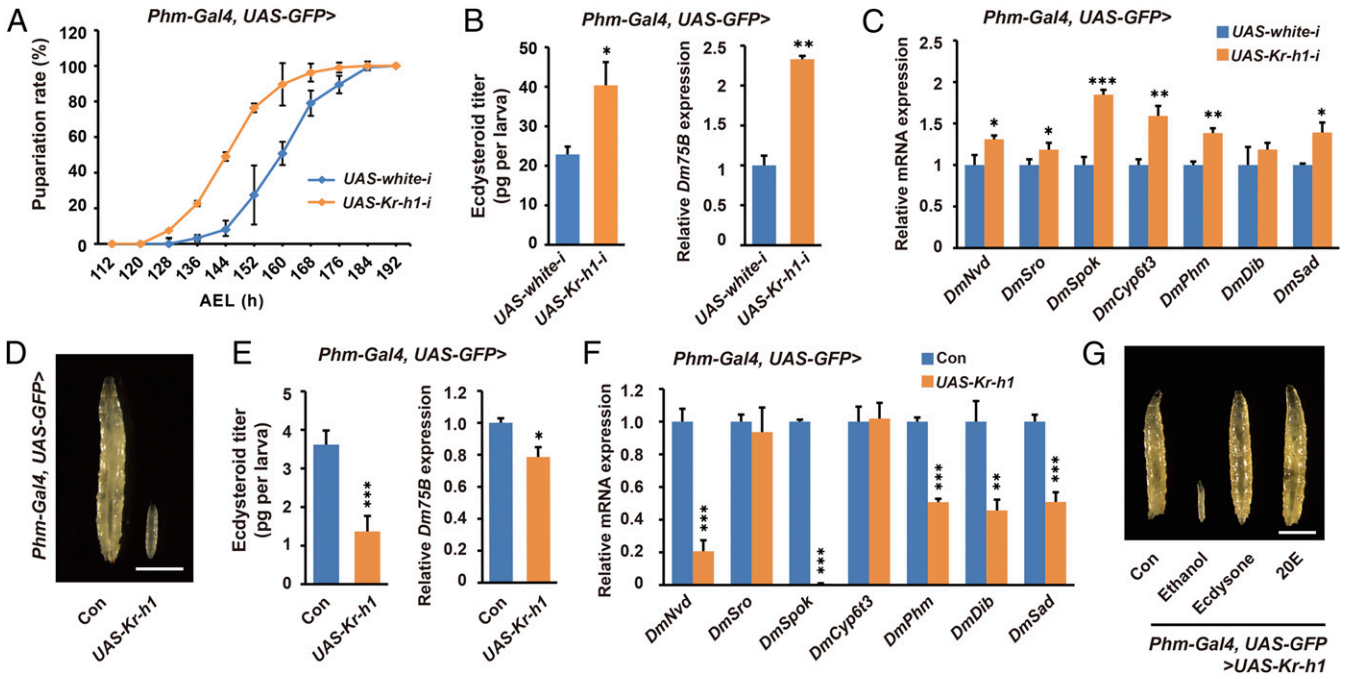

Fig. 2. Level of *DmKr-h1* expression in *Drosophila* PG affects larval development, ecdysone biosynthesis and transcription of steroidogenic enzyme genes. (*A*) Pupariation rate with knockdown of *DmKr-h1* in *Drosophila* PG driven by *Phm-Gal4* compared with control (*white-i*). (*B*) Measurement of both ecdysone titer in the whole body and *DmE75B* mRNA level in the fat body of *DmKr-h1* knockdown larvae and control larvae at 96 h AEL. (*C*) *DmKr-h1* knockdown in *Drosophila* PG up-regulates expression of steroidogenic enzyme genes in the brain–RG complex of larvae at 96 h AEL. (*D*) PG-specific overexpression of *DmKr-h1* leads to developmental arrest at the first-larval (L1) instar. (Scale bar, 2.5 mm.) (*E*) PG-specific *DmKr-h1* overexpression-mediated inhibition of ecdysone biosynthesis in the PG and *DmE75B* expression in the fat body at 48 h AEL. (*F*) Fold-change in steroidogenic enzyme gene expression in brain–RG complex overexpressing *DmKr-h1* relative to control at 48 h AEL. (*G*) Feeding larvae with ecdysone or 20E rescued developmental L1 arrest induced by *DmKr-h1* overexpression in the PG. (Scale bar, 2 mm.) Ecdysteroid feeding was started at 48 h AEL. All experiments were performed in three biological replicates. Values are represented as the mean ± SE (error bars). For the significance test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control.

Drosophila Spok and its *Bombyx* ortholog Spo are potential rate-limiting enzymes involved in insect ecdysteroidogenesis (24, 26–28). To characterize the KBSs present in the *Drosophila DmSpok* promoter, we inserted different 5′-flanking truncated fragments of the *DmSpok* promoter into pGL3-Basic vector and performed luciferase reporter assays in *Drosophila* S2 cells. Compared with the control, *DmKr-h1* overexpression inhibited the activity of *DmSpok* promoter truncations that include the distal and proximal KBSs, and this inhibition disappeared when the proximal KBS was truncated (Fig. 3*C*). Additional analysis revealed that either deletion or mutation of the proximal KBS in the *DmSpok* promoter resulted in a loss of promoter activity inhibition by DmKr-h1 (Fig. 3*D*). Remarkably, consistent with observations in *Drosophila*, inhibition of *Bombyx BmSpo* promoter activity by BmKr-h1 was also eliminated following deletion or mutation of the proximal KBS in the *BmSpo* promoter (Fig. 3*E*). These results strongly indicate that Kr-h1 directly and negatively regulates transcription of steroidogenic enzymes.

Kr-h1 Directly Binds to the Promoters of Steroidogenic Enzyme Genes. We next examined the possibility that Kr-h1 binds directly to the promoters of *Drosophila DmSpok* and *Bombyx BmSpo*. First, we performed chromatin immunoprecipitation (ChIP) to check the direct binding of DmKr-h1 on the proximal KBS of the *DmSpok* promoter. V5-tagged DmKr-h1 overexpression vector was constructed and then transfected into *Drosophila* S2 cells. Subsequent genomic PCR analysis using a specific primer pair covering the proximal KBS demonstrated that compared with nonspecific IgG antibody as a negative control, the V5-antibody targeting tagged DmKr-h1 could specifically immunoprecipitate DNA regions covering the proximal KBS of the *DmSpok* promoter (Fig. 4*A*).

Next, based on the proximal KBS in the *DmSpok* promoter, we designed a specific biotinylated probe and carried out an EMSA. The results revealed that purified recombinant DmKr-h1 protein could bind to the biotinylated probes in a dose-dependent manner, and that this binding could be competitively inhibited by unlabeled cold probes (Fig. 4*B*). However, unlabeled probes with either mutation or deletion of KBS could not compete for DmKr-h1 binding to the biotinylated probes (Fig. 4*C*). In addition, compared with IgG antibody as a negative control, coincubating nucleoproteins extracted from S2 cells overexpressing V5-tagged DmKr-h1 with V5-antibody caused a loss of DmKr-h1 binding to the biotinylated probes against KBS (Fig. S7). Furthermore, similar examination in *Bombyx* also revealed that BmKr-h1 could directly bind to the proximal KBS motif in the *BmSpo* promoter (Fig. 4 *D–F*). These findings collectively suggest that Kr-h1 inhibits transcription of steroidogenic enzyme genes by directly binding to their promoters.

Kr-h1 Induces DNA Methylation of the Promoter of Steroidogenic Enzyme. Studies in both *Drosophila* and mammals have shown that Krüppel and Krüppel-like factor family members containing zinc-finger domains can epigenetically induce DNA methylation of the promoters of their target genes to inhibit their transcription (29, 30). Therefore, we investigated whether DmKr-h1 could induce DNA methylation at cytosines (C) around the proximal KBS present in the *DmSpok* promoter by using the bisulfite sequencing PCR (BSP) approach. Interestingly, our BSP analysis showed that cytosines located in the ∼200-bp region upstream of the proximal KBS in the *DmSpok* promoter were methylated following *DmKr-h1* overexpression (Fig. 4*G* and Fig. S8). Methylation of these cytosines was eliminated by addition of specific DNA methylation inhibitor 5-Aza-2,9-deoxycytidine (Aza) (Fig. 4*G*). Furthermore,

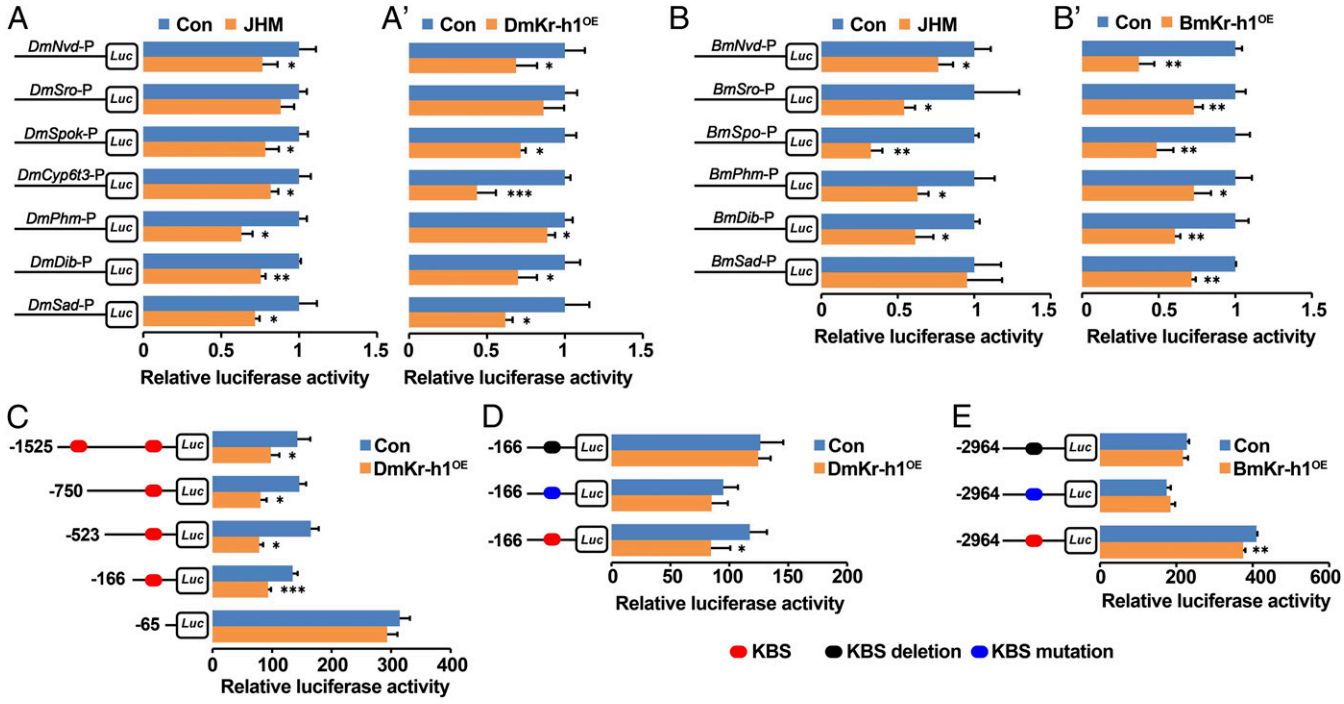

Fig. 3. Kr-h1 inhibits the activities of the *Drosophila* and *Bombyx* steroidogenic enzyme promoters. (*A* and *A′*) JHM application and *DmKr-h1* overexpression represses the activity of steroidogenic enzyme promoters in *Drosophila*. Luciferase activity fold-change values were normalized to the control. (*B* and *B′*) JHM application and *BmKr-h1* overexpression repress activity of steroidogenic enzyme promoters in *Bombyx*. (*C*) DmKr-h1 affects the luciferase activity driven by different truncations of the *DmSpok* promoter. DmKr-h1 did not show transcriptional inhibition activity when the KBS of the *DmSpok* promoter was truncated. (*D*) Effects of DmKr-h1 on the luciferase activity driven by *DmSpok* promoters with either a deletion or a mutation form of proximal KBS. (*E*) Effects of BmKr-h1 on the luciferase activity driven by *BmSpo* promoters with either a deletion or a mutated form of KBS. All experiments were performed in three biological replicates. Values are represented as the mean + SE (error bars). For the significance test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control.

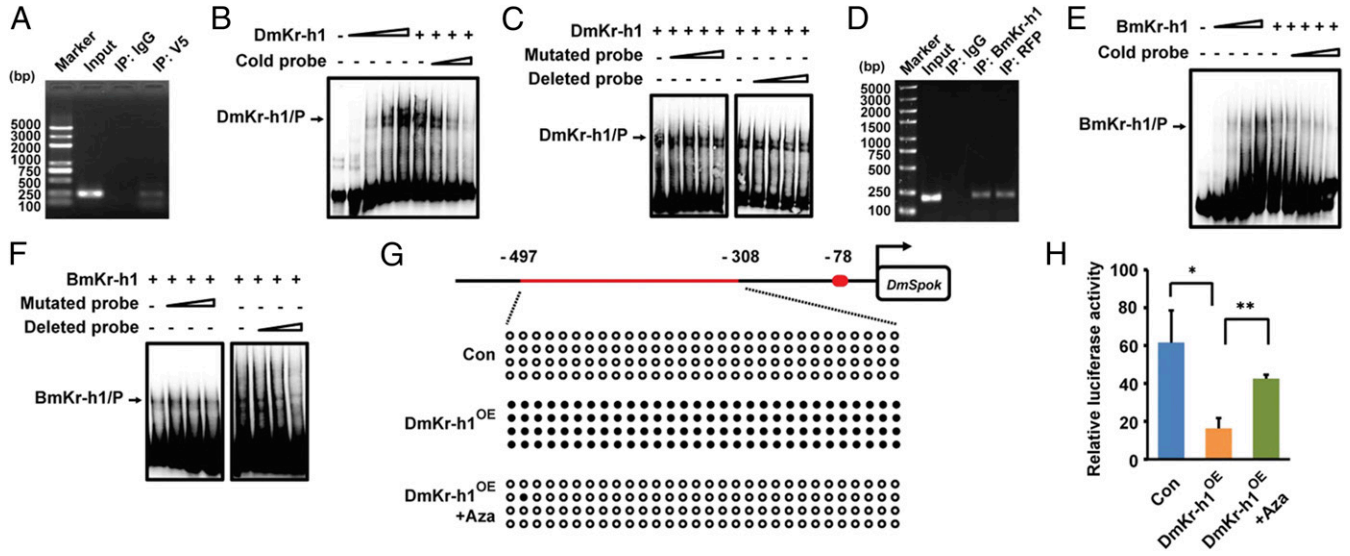

Fig. 4. Kr-h1 directly binds the KBS in *DmSpok* and *BmSpo* promoters to induce DNA methylation. (*A*) ChIP-PCR assay in *Drosophila* S2 cells with DmKr-h1 overexpression. Specific primers covering proximal KBS of the *DmSpok* promoter were used. (*B* and *C*) EMSA confirmed that DmKr-h1 directly binds to the proximal KBS. Recombinant DmKr-h1 protein binds to the biotinylated probes covering the proximal KBS in the *DmSpok* promoter in a dose-dependent manner; this binding can be competitively inhibited by unlabeled cold probes (*B*). Unlabeled probes with either KBS mutation or KBS deletion cannot compete for binding of recombinant DmKr-h1 protein to the biotinylated probes (*C*). (*D*) ChIP-PCR assay of the direct binding of overexpressed BmKr-h1 to the proximal KBS of the *BmSpo* promoter *in Bombyx* BmE cells. (*E* and *F*) EMSA confirms that recombinant BmKr-h1 directly binds to the proximal KBS of the *BmSpo* promoter. (*G*) Schematic diagram of methylated cytosine present in the *DmSpok* promoter after DmKr-h1 overexpression in S2 cells. Filled circles, methylated cytosines; empty circles, unmethylated cytosines; red rounded rectangle, proximal KBS. (*H*) The DNA methylation inhibitor Aza rescued the inhibitory activity of DmKr-h1. All experiments were performed in three biological replicates. Values are represented as the mean + SE (error bars). For the significance test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control.

we analyzed the effects of DmKr-h1–induced DNA methylation on the activity of the *DmSpok* promoter. Our luciferase reporter assay confirmed that DmKr-h1–associated inhibition on transcriptional activity of *DmSpok* promoter was potently abolished by the treatment with the DNA methylation inhibitor Aza (Fig. 4*H*). Taken together, our results indicate that direct repression of DmKr-h1 on the transcription of *DmSpok* is most likely caused by Kr-h1–induced DNA methylation of the *DmSpok* promoter following its direct binding to the KBS.

Kr-h1 Has No Effect on DNA Replication in PG Cells. Generally, organ growth and size are associated with organ functions in animals (31). In specific organs, endoreplication is further associated with normal organ size and function (32). A previous report in *Drosophila* has shown that defective endoreplication in PG cells reduces PG size and ecdysone biosynthesis (33). Thus, we next examined the effects of DmKr-h1 on DNA replication in PG cells by EdU staining. Reduction in *DmKr-h1* levels, via either PG-specific knockdown of *DmKr-h1* or in *DmKr-h1* heterozygotes, did not affect either DNA replication in the PG or PG size at 96 h AEL (Fig. S9 *A* and *B*). Similarly, PG-specific overexpression of *DmKr-h1* did not alter DNA replication at 48 h AEL (Fig. S9*C*). Taken together, these results suggest that DmKr-h1 is not involved in DNA replication in *Drosophila* PG cells.

Because PG-specific *DmKr-h1* overexpression in *Drosophila* resulted in developmental L1 arrest and inhibited ecdysone production (Fig. 2 *D* and *E*), we speculated that the size of the PG in larvae overexpressing *DmKr-h1* should be reduced. As expected, at 96 h AEL, the size of the PG from *Drosophila* larvae with PG-specific *DmKr-h1* overexpression is smaller than the control (Fig. S9*D*), being similar to that at 48 h AEL (Fig. S9*C*). However, DNA replication in PG cells was also not affected at this stage (Fig. S9*D*). Interestingly, feeding exogenous 20E to *Drosophila* larvae with *DmKr-h1* overexpression in the PG, recovered normal developmental transition and body size (Fig. 2*G*), as well as PG size comparable to the control (Fig. S9*D*). These data together suggest that PG-specific *Kr-h1* overexpression-reduced ecdysone production appears to directly cause developmental L1 arrest, which in turn disrupts systemic growth of the body and organs, including the PG.

Discussion

JH and ecdysone exhibit periodic, opposite changes in hemolymph titers and play antagonistic roles during insect growth and development, indicating an essential cross-talk between these two endocrine hormones (1, 2). A striking finding of our study is that Kr-h1 attenuates ecdysone production in the PG by directly repressing transcription of steroidogenic enzymes in both *Drosophila* and *Bombyx*. This conclusion is supported by: (*i*) JH directly acts on the PG to induce *Kr-h1* expression; (*ii*) Kr-h1 expression in the PG inhibits the transcription of steroidogenic enzymes and ecdysone biosynthesis; (*iii*) Kr-h1 directly binds to the promoters of steroidogenic enzymes and represses their activities. Notably, previous reports have demonstrated that the nuclear receptor DHR4 also acts as a repressor of ecdysone biosynthesis and larval–pupal transition (25, 34), indicating a potential cross talk between DHR4 and Kr-h1. Given that the mechanism by which Kr-h1 is regulated is largely unknown, future studies will be required to elucidate the relationship between DHR4 and Kr-h1 in insect PG regarding ecdysone biosynthesis control.

The regulatory mechanism underlying transcriptional repression of ecdysone action by JH signaling is particularly intriguing. Even though DNA methyltransferases and other factors directly involved in DNA methylation are not well characterized in *Drosophila*, genomic DNA methylation and its associated transcriptional suppression have been extensively reported in embryo, larva, and adult stages (29, 35, 36). Our results in *Drosophila* S2 cells indicate that chemical inhibition of DNA methylation is sufficient to alleviate Kr-h1–associated transcriptional suppression of the steroidogenic enzyme gene *Spok*, suggesting that Kr-h1 suppresses ecdysone enzyme gene transcription via DNA methylation. Thus, identification of DNA methyltransferases and other factors that are possibly involved in Kr-h1–associated DNA methylation

and transcriptional suppression of steroidogenic enzymes in larval PG will be another important area of investigations.

Previous studies in *Drosophila* have found that knockdown of genes, such as *TSC1/2* and *Fzr*, reduce PG size and ecdysone production (33, 37), therefore raising the possibility that PG size may determine ecdysone biosynthesis. However, a recent report has demonstrated that although endoreplication progression in *Drosophila* PG is nutrient-dependent and required for PG growth, the lack of ecdysone production caused by endoreplication inhibition cannot be rescued by genetically increasing PG size (33), suggesting that PG size is probably not the major factor affecting ecdysone biosynthesis. Our data indicate that the level of Kr-h1 in *Drosophila* PG has no effect on DNA replication required for endoreplication progression-mediated PG growth, and that exogenous 20E can completely rescue PG-specific *Kr-h1* overexpression-induced arrest in developmental transition and PG growth. Because ecdysone limits systemic growth, whereas ecdysone-triggered larval molting is essential for systemic growth (1, 38, 39), we propose that developmental arrest caused by Kr-h1–induced reduction in ecdysone biosynthesis in insect PG initially disrupts systemic growth of the body and the PG.

In summary, we have shown that in both *Drosophila* and *Bombyx*, the transcription factor Kr-h1 as a key JH signal transducer binds directly to specific *cis*-regulatory elements in the promoters of steroidogenic enzyme genes (i.e., KBSs), and induces DNA methylation of the promoters, which in turn inhibits transcription of steroidogenic enzymes and negatively regulates ecdysone biosynthesis (Fig. S10). Taken together, these results point to a mechanism by which Kr-k1–mediated JH signaling in the PG exerts an evolutionary conserved repressive role on ecdysone biosynthesis during insect growth and development.

Materials and Methods

A detailed description of the materials and methods is provided in *SI Materials and Methods*. Briefly, gene expression was profiled via qRT-PCR, RNA in situ hybridization, or immunostainings. Genetic manipulation of *Drosophila Kr-h1* gene was performed. Ecdysteroid titer was estimated by EIA. Direct regulation of Kr-h1 on the transcription of steroidogenic enzymes were analyzed by luciferase reporter analyses, ChIP, and EMSA experiments. BSP analysis was applied to detect DNA methylation.

Note. Please note that as we were preparing the submission of our manuscript, we noticed that Liu et al. (40) also found that Kr-h1 in the PG inhibits ecdysteriod biosynthesis.

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