Histone H3K27 methylation–mediated repression of *Hairy* regulates insect developmental transition by modulating ecdysone biosynthesis

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Insect development is cooperatively orchestrated by the steroid hormone ecdysone and juvenile hormone (JH). The polycomb repressive complex 2 (PRC2)–mediated histone H3K27 trimethylation (H3K27me3) epigenetically silences gene transcription and is essential for a range of biological processes, but the functions of H3K27 methylation in insect hormone action are poorly understood. Here, we demonstrate that H3K27 methylation–mediated repression of *Hairy* transcription in the larval prothoracic gland (PG) is required for ecdysone biosynthesis in *Bombyx* and *Drosophila*. H3K27me3 levels in the PG are dynamically increased during the last larval instar. H3K27me3 reduction induced by the down-regulation of PRC2 activity via inhibitor treatment in *Bombyx* or PG-specific knockdown of the PRC2 component *Su(z)12* in *Drosophila* diminishes ecdysone biosynthesis and disturbs the larval–pupal transition. Mechanistically, H3K27 methylation targets the JH signal transducer *Hairy* to repress its transcription in the PG; PG-specific knockdown or overexpression of the *Hairy* gene disrupts ecdysone biosynthesis and developmental transition; and developmental defects caused by PG-specific *Su(z)12* knockdown can be partially rescued by *Hairy* down-regulation. The application of JH mimic to the PG decreases both H3K27me3 levels and *Su(z)12* expression. Altogether, our study reveals that PRC2-mediated H3K27 methylation at *Hairy* in the PG during the larval period is required for ecdysone biosynthesis and the larval–pupal transition and provides insights into epigenetic regulation of the crosstalk between JH and ecdysone during insect development.

H3K27 methylation | *Hairy* | ecdysone biosynthesis | juvenile hormone | repression

Insect developmental transitions are cooperatively mediated by two major endocrine hormones, the steroid hormone ecdysone and the sesquiterpenoid juvenile hormone (JH) (1–3). Ecdysone is highly produced in the prothoracic gland (PG) at the end of each larval instar and directly triggers larval molting or metamorphosis with the larval–pupal transition (2, 4). JH is massively synthesized in corpora allata at the early stage of each larval instar and functions as an antimetamorphic hormone to maintain insect larval growth and to prevent larvae from undergoing a developmental transition (3, 5–7). Recent studies have revealed that ecdysone and JH can mutually repress their biosynthesis by inhibiting the transcription of the enzymes involved in biosynthetic pathways (5–7), and the JH signaling pathway negatively regulates the transcription of the genes involved in ecdysone signaling (8, 9). Undoubtedly, transcriptional repression should be a key mechanism underlying the dynamic concentration changes and antagonistic actions of these two hormones during insect development.

Histone modifications play essential roles in the regulation of the chromatin structure and gene expression in eukaryotes. Increasing evidence have demonstrated that the trimethylation of histone H3 lysine 27 (H3K27me3) generally functions as a repressive epigenetic marker to silence gene transcription (10–12). H3K27 methylation is catalyzed by the polycomb repressive complex 2 (PRC2) with histone methyltransferase activity (12–17). PRC2 contains four core components, namely Enhancer of zeste [E(z)], Suppressor of zeste 12 [Su(z)12], Extra sex combs (Esc), and Nurf55 (also known as chromatin assembly factor 1 p55 subunit, Caf1-55), and is conserved in terms of both sequence and function in eukaryotes (14, 15, 18, 19). PRC2-mediated H3K27 methylation mechanically causes the compaction of the related chromatin regions at target gene loci and subsequently represses target gene expression (14, 16, 20–22).

PRC2-mediated H3K27 methylation is associated with various developmental processes in insects. In *Drosophila*, the PRC2 deficiency–induced decrease in H3K27me3 levels at different target genes leads to defects in body segmentation (20, 23, 24), induces sterility by switching the oocyte fate (25), and prolongs a healthy lifespan by promoting glycolysis in adults (26). Moreover, the increase in H3K27me3 levels caused by the mutation of the *UTX* gene encoding a H3K27me3 demethylase not only disrupts salivary gland degradation during the prepupal period by decreasing the transcription of apoptosis-related genes (27) but also destroys the architecture of adult testis niche by dysregulating JAK-STAT signaling (28). Recently, a genome-wide RNA interference (RNAi)

Significance

PRC2-mediated H3K27 methylation functions in epigenetic transcriptional repression and is involved in various developmental processes in eukaryotes. Insect developmental transitions are regulated by juvenile hormone (JH) and ecdysone in an antagonistic manner. Here, we used *Bombyx* and *Drosophila* to show that PRC2-mediated H3K27 methylation at the *Hairy* gene involved in JH signaling suppresses *Hairy* transcription in the prothoracic gland (PG), which is required for steroidogenic enzyme transcription, ecdysone biosynthesis, and the larval–pupal transition. JH impairs PRC2-mediated H3K27 methylation in the PG. Our findings establish an essential role for PRC2-mediated H3K27 methylation in hormone-mediated developmental transitions and further deepen our understanding of epigenetic regulatory mechanisms underlying the antagonistic actions of JH and ecdysone in insects.

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screen in the *Drosophila* PG revealed that knockdown of the PRC2 component leads to a delay in the larval–pupal transition (29), raising a possibility that PRC2-mediated H3K27 methylation in the PG might be epigenetically involved in the control of ecdysone biosynthesis.

In this study, we found that PRC2-mediated H3K27 methylation in the PG of *Bombyx* and *Drosophila* larvae regulates ecdysone biosynthesis. H3K27me3 levels are progressively elevated in the PG during the last larval instar. The PRC2 activity impairment–induced decrease in H3K27me3 levels in the PG reduces ecdysone biosynthesis and disrupts the larval–pupal transition. Mechanistically, we showed that H3K27 methylation targets the *Hairy* gene that is involved in JH signaling to inhibit its transcription and thereby abolishes the repression of Hairy on ecdysone biosynthesis. JH negatively regulates H3K27 methylation by affecting the transcription of the PRC2 component *Su(z)12* in the PG. Collectively, our findings uncover an epigenetic mechanism by which PRC2-mediated histone H3K27 methylation in the PG impairs JH inhibition of insect ecdysone biosynthesis.

Results

Dynamic Changes of H3K27me3 Levels in the PGs of *Bombyx* and *Drosophila* Larvae. To determine the role of PRC2-mediated H3K27 methylation in insect larval development, we first examined dynamic changes of H3K27me3 levels in the PG of both *Bombyx* and *Drosophila* individuals during larval–pupal transition. The PGs were separately isolated from *Bombyx* larvae at four developmental stages, including the first day of the last (fifth) larval instar (L5D1), third day (L5D3), fifth day (L5D5), and just wandering (W0). An immunostaining analysis and a Western blot experiment with an anti-H3K27me3 antibody showed that H3K27me3 levels were slightly low in *Bombyx* PG at the early stage of the last larval instar and progressively increased until reaching a high level at the just wandering stage (Fig. 1*A* and *SI Appendix*, Fig. S1 *A* and *B*). In addition, during *Drosophila* larval–pupal transition, low and high H3K27me3 levels were detected in the PG of the brain–ring gland (RG) complex at 84 h after egg laying (AEL; the early stage of the last larval instar) and at 132 h AEL (pupariation), respectively (Fig. 1*B* and *SI Appendix*, Fig. S1*C*).

We further investigated the expression patterns of PRC2 core components in *Bombyx* PG that could be completely isolated from larval individual. RT-qPCR analysis revealed that the messenger RNA (mRNA) expression of two PRC2 components, *Su(z)12* and *E(z)*, exhibited a progressive increase during the last larval instar, and the other two components were highly expressed at the early stage (*SI Appendix*, Fig. S2 *A–D*). In addition, *Drosophila Su(z)12* expression in the brain–RG complex containing the PG exhibited an obvious increase before pupariation (*SI Appendix*, Fig. S2 *E–H*). Collectively, our results showed that the dynamics of both H3K27me3 levels and the PRC2 component expressions were similar to the temporal changes in ecdysone titer during the larval–pupal transition.

Disruption of H3K27 Methylation in Larval PG Affects the Larval–Pupal Transition and Ecdysone Biosynthesis in *Bombyx* and *Drosophila*. We next analyzed the effects of PRC2-mediated H3K27 methylation in the PG on insect larval development. First, we treated *Bombyx* larvae on the fifth day of the last larval instar with GSK126, an inhibitor of PRC2 activity (30), and found that compared with DMSO treatment as a control, GSK126 treatment decreased H3K27me3 levels in the PG but had no effect on the levels of the other two histone methylation modifications, H3K36me3 and H3K4me3 (*SI Appendix*, Fig. S3 *A–D*). Obviously, GSK126 treatment delayed *Bombyx* larval–pupal transition (Fig. 2*A* and *SI Appendix*, Fig. S3 *E* and *F*). Furthermore, we performed RNAi-mediated knockdown of four core PRC2 components, including *E(z)*, *Su(z)12*, *Esc*, and *Nurf55*, in the PG of *Drosophila* larvae using the PG-specific driver Phm-Gal4. The results revealed that knockdown of the *Su(z)12* gene, using two RNAi lines targeting

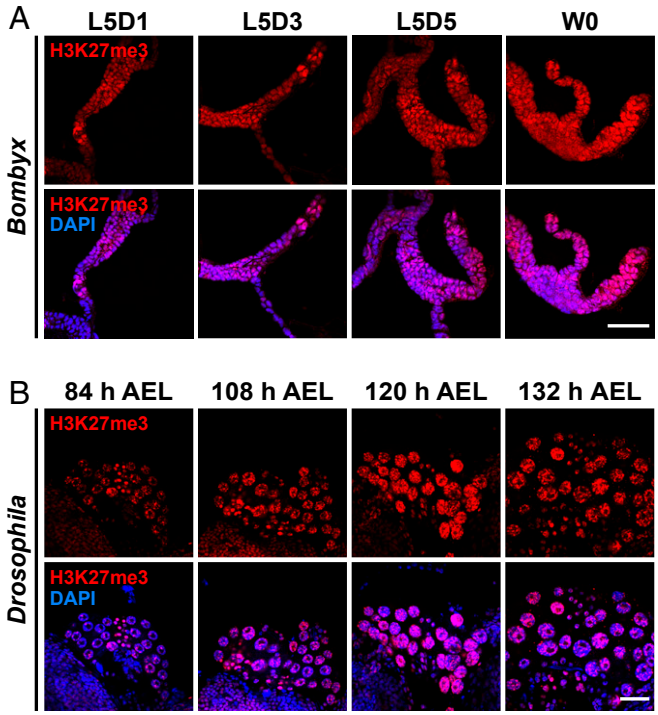

Fig. 1. Developmental dynamics of H3K27me3 levels in the PGs of both *Bombyx* and *Drosophila* larvae. (*A*) Dynamic change of H3K27me3 levels in *Bombyx* PG during the last (fifth) larval instar. L5D1, the first day of the fifth larval instar; L5D3, the third day of the fifth larval instar; L5D5, the fifth day of the fifth larval instar; W0, just wandering. (Scale bar, 200 μm.) (*B*) Dynamic change of H3K27me3 levels in *Drosophila* PG during the last (third) larval instar. (Scale bar, 20 μm.)

distinct sequences, caused a developmental defect in the larval–pupal transition and formed a large permanent L3 (the third instar) larvae (Fig. 2*B* and *SI Appendix*, Fig. S4 *A–C*). As expected, H3K27me3 levels were eliminated in the PG following PG-specific *Su(z)12* knockdown (*SI Appendix*, Fig. S4 *D* and *E*). Other than *Su(z)12*, knockdown of other PRC2 components in the PG did not affect larval–pupal transition (*SI Appendix*, Fig. S4 *F* and *G*). These data demonstrate that PRC2-mediated H3K27 methylation in the PG of *Bombyx* and *Drosophila* larvae is required for the larval–pupal transition.

Because insect ecdysone is produced in the PG during the larval period and functions to trigger developmental transitions (2, 4), we next investigated whether ecdysone biosynthesis could be affected by changes in PRC2-mediated H3K27 methylation. Compared with the control, both the application of GSK126 to *Bombyx* larvae and PG-specific knockdown of *Su(z)12* in *Drosophila* larvae impaired ecdysone production (Fig. 2 *C* and *D*), down-regulated the transcription of the ecdysone-responsive *E75B* gene in the ecdysone-targeting fat body (*SI Appendix*, Fig. S5), and reduced the expression of steroidogenic enzyme genes, including *Nvd*, *Sro*, *Spo*/*Spok*, and *Sad*, in the PG (Fig. 2 *E–G* and *SI Appendix*, Fig. S4*H*). In addition, the application of GSK126 to ex vivo–cultured *Bombyx* PG or *Drosophila* brain–RG complex containing the PG also led to a decrease in both H3K27me3 levels and steroidogenic enzyme transcription (*SI Appendix*, Fig. S6). Importantly, feeding 20-hydroxyecdysone (20E), an active derivative of ecdysone, partially rescued the defect in the larval–pupal transition caused by PG-specific *Su(z)12* knockdown in *Drosophila* or by GSK126 treatment in *Bombyx* (Fig. 2*H* and *SI Appendix*, Fig. S7). Taken together, our results suggest that the larval–pupal transition defect caused by the disruption of H3K27 methylation in the PG was due to a reduction in ecdysone production.

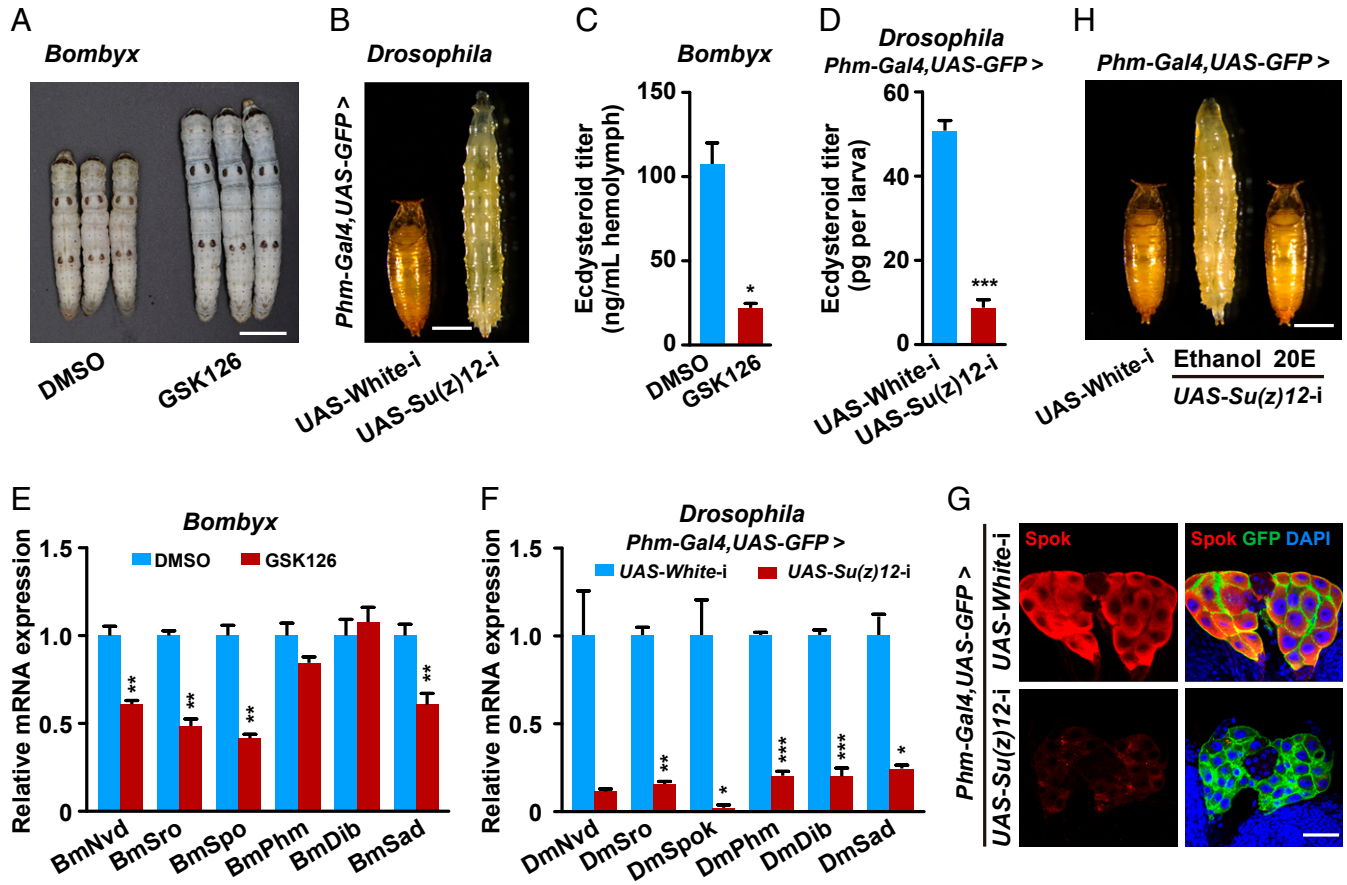

Fig. 2. Reduction in H3K27me3 levels in the PG disturbs larval–pupal transition and impairs ecdysone production and the expression of steroidogenic enzymes. (*A*) Developmental change of *Bombyx* larvae following treatment with GSK126 as an inhibitor of PRC2 activity. GSK126 (50 μg per larva) was injected into *Bombyx* larvae on the fifth day of the fifth larval instar. DMSO treatment was used a control. (Scale bar, 1 cm.) (*B*) PG-specific knockdown of PRC2 core component *Su(z)12* in *Drosophila* larvae arrested larval–pupal transition. The *Su(z)12* RNAi stock (THU5817) was obtained from the Tsinghua Fly Center. (Scale bar, 1 mm.) (*C* and *D*) Changes in ecdysone titer in the hemolymph of *Bombyx* larvae after GSK126 application and in the whole body of *Drosophila* larvae with PG-specific *Su(z)12* knockdown. The hemolymph was collected from *Bombyx* larvae at 24 h after GSK126 treatment and the whole *Drosophila* larvae at 120 h AEL, respectively. (*E–G*) Changes in steroidogenic enzyme expression in the PG of *Bombyx* larvae following GSK126 treatment (*E*) and of *Drosophila* larvae with PG-specific *Su(z)12* knockdown (*F* and *G*). (Scale bar, 20 μm.) (*H*) Feeding 20E as an active derivative of ecdysone partially rescued the developmental defects caused by PG-specific *Su(z)12* knockdown. Larvae with PG-specific *Su(z)12* knockdown were separately supplied 20E and ethanol at 96 h AEL. (Scale bar, 1 mm.) All experiments were conducted with three biological replicates. The values are represented as the mean ± SE (error bars). For the significance test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus the control.

The JH Signal Transducer *Hairy* Is a Target of H3K27 Methylation in the PG of *Bombyx* and *Drosophila* Larvae. PRC2-mediated H3K27 methylation generally blocks the transcription of its target genes (16, 24, 31, 32). Because H3K27 methylation in the PG is positively associated with ecdysone biosynthesis in insects, we reasoned that H3K27 methylation might target the genes that function as repressors of ecdysone biosynthesis. To identify H3K27me3 targets related to ecdysone biosynthesis in the PG during the last larval instar, we performed a chromatin immunoprecipitation sequencing (ChIP-seq) analysis in *Bombyx* PG at the L5D1 stage without ecdysone production and the W0 stage with high ecdysone titer using an anti-H3K27me3 antibody. A comparative analysis identified 2,089 differential peaks between L5D1 and W0, 1,506 of which were highly present at the W0 stage and associated with 650 genes (Dataset S1 and *SI Appendix*, Fig. S8). Further analysis revealed that 465 genes were specifically marked by W0-biased H3K27me3 peaks and most of them enriched in the Gene Ontology (GO) categories of nucleic acid binding and transcription regulator activity (*SI Appendix*, Fig. S8 and Dataset S1).

Intriguingly, we observed that the *Hairy* gene, which encodes a basic helix–loop–helix (bHLH) transcription factor that functions as a key transducer in the JH repression hierarchy (33–36), was included in the collection of 62 transcription factors as potential H3K27me3 targets in *Bombyx* PG at the W0 stage (*SI Appendix*, Fig. S8 and Dataset S1). The identified H3K27me3 ChIP peak was located within the gene body of *BmHairy* (Fig. 3*A* and *SI Appendix*, Fig. S8 and Dataset S1). Given that enhancers involved in the regulation of gene transcription are generally located within the gene body or outside the promoter and inactive enhancers are mainly marked by H3K27me3 (37–39), we proposed that H3K27me3 may modulate the activity of a linked enhancer within the gene body of *BmHairy*. In addition, ChIP-seq data from whole bodies of *Drosophila* larvae at the third instar revealed the presence of H3K27 methylation within the potential promoter of *DmHairy* (Fig. 3*B*). Furthermore, a ChIP followed by qPCR (ChIP-qPCR) assay confirmed that H3K27me3 levels at the *BmHairy* locus in *Bombyx* PG were higher at the W0 stage than that at the L5D1 stage (Fig. 3*C*). Consistently, H3K27me3 enrichment within the *DmHairy* promoter in the *Drosophila* brain–RG complex containing the PG at 120 h AEL could be significantly diminished by PG-specific *Su(z)12* knockdown (Fig. 3*D*).

We further investigated the effects of PRC2-mediated H3K27 methylation on the expression of the *Hairy* gene in the PG of *Bombyx* and *Drosophila* larvae. An RT-qPCR analysis revealed that *Hairy* expression was high at the early stage of the last larval

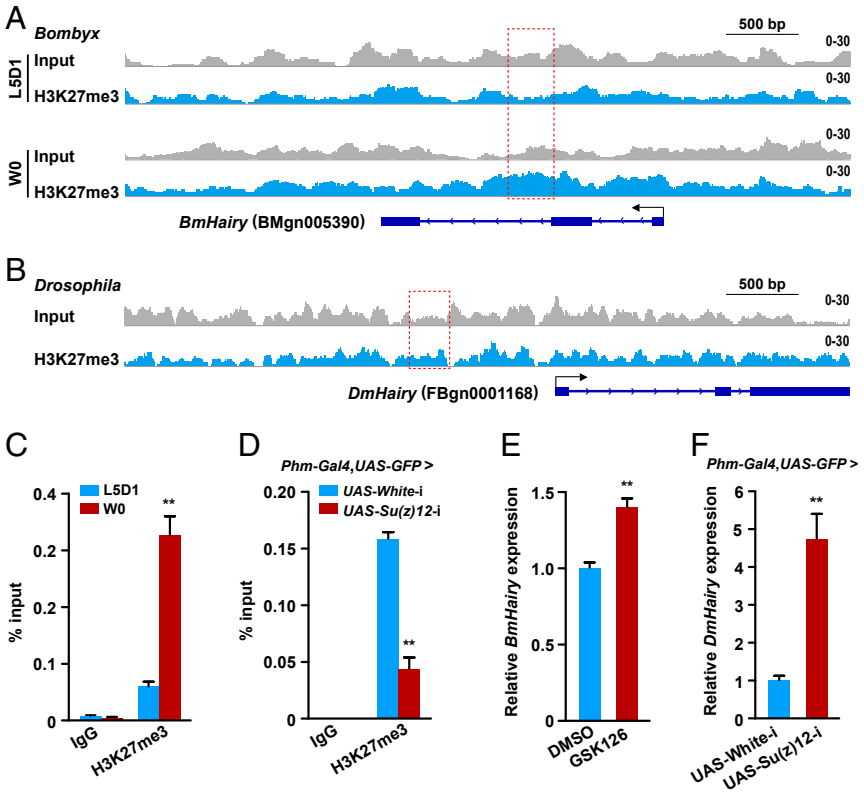

Fig. 3. H3K27me3 targets the *Hairy* gene to repress its transcription. (*A* and *B*) ChIP-seq analysis identified the *Hairy* gene involved in JH signaling as a target of PRC2-mediated H3K27 methylation in the PG of *Bombyx* larvae (*A*) and in the whole body of *Drosophila* larvae (*B*). The H3K27me3 ChIP peak at the *Hairy* locus is outlined by dashed frame. L5D1, the first day of the fifth larval instar; W0, just wandering. (*C*) ChIP-qPCR analysis of the PG of *Bombyx* larvae using anti-H3K27me3 antibody showed dynamic H3K27me3 enrichment at the *Hairy* locus. The anti-IgG antibody was used as a negative control. (*D*) PG-specific *Su(z)12* knockdown in *Drosophila* larvae caused a decrease in H3K27me3 level at the *Hairy* locus at 120 h AEL. (*E*) GSK126 treatment to *Bombyx* larvae up-regulated *Hairy* expression in the PG. (*F*) PG-specific *Su(z)12* knockdown in *Drosophila* larvae increased *Hairy* expression in the brain–RG complex containing the PG. All experiments were conducted with three biological replicates. The values are represented as the mean ± SE (error bars). For the significance test, $^{**}P < 0.01$ versus the control.

instar and progressively decreased until it reached a low level at the W0 stage in *Bombyx* or at 132 h AEL in *Drosophila* (*SI Appendix*, Fig. S9), which was consistent with the temporal change in JH titer in the hemolymph (3) but was contrary to the dynamic of H3K27me3 levels in the PG (Fig. 1 *A* and *B*). In addition, the reduction in H3K27me3 levels in the PG caused by either GSK126 treatment in *Bombyx* larvae or PG-specific *Su(z)12* knockdown in *Drosophila* larvae led to an elevation of *Hairy* transcription in the PG (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S10). Collectively, our results indicate that PRC2-mediated H3K27 methylation in the PG of insect larvae before pupation targets the *Hairy* gene to repress its transcription.

Hairy Represses Ecdysone Biosynthesis by Down-Regulating the Transcription of Steroidogenic Enzymes. To examine whether Hairy expression in the PG is involved in ecdysone-mediated larval–pupal transition, we first performed RNAi-mediated knockdown of the *DmHairy* gene in *Drosophila* PG. As a result, compared with the control, PG-specific *Hairy* knockdown caused precocious larval–pupal transition (Fig. 4*A*), induced a rapid increase of ecdysone titer in the hemolymph (Fig. 4*B*), and up-regulated the expression of both the ecdysone-responsive gene *E75B* in the ecdysone-targeted fat body and steroidogenic enzymes in the PG (Fig. 4*C* and *SI Appendix*, Fig. S11 *A–C*). Similarly, we analyzed the effects of *Hairy* mutant using a point mutation allele h^{22} and found that compared with the wild-type control, the heterozygous mutation in *Hairy* also caused precocious pupariation and an increase in ecdysone production (*SI Appendix*, Fig. S11 *D* and *E*).

We further checked the effects of *Hairy* overexpression in the PG on the developmental transition in *Drosophila*. To avoid possible arrest at the early stage of the larval period caused by *Hairy* overexpression, the *Tub-gal80^{ts};Phm-Gal4* driver was used to drive *Hairy* overexpression in the PG of *Drosophila* larvae at the beginning of the third instar. Compared with the control, *DmHairy* overexpression in the PG resulted in developmental arrest at the third larval instar (Fig. 4*D*). Ecdysone production and the expression of both steroidogenic enzymes in the PG and *E75B* in fat body were reduced following PG-specific *DmHairy* overexpression (Fig. 4 *E* and *F* and *SI Appendix*, Fig. S11 *F–H*). Further luciferase reporter assay and ChIP-PCR experiments in *Bombyx* BmE cells and *Drosophila* S2 cells confirmed that Hairy negatively regulated the transcription of steroidogenic enzyme gene *BmSpo*/*DmSpok* by directly binding to its promoter (*SI Appendix*, Fig. S12). Importantly, the phenotype of developmental arrest caused by *DmHairy* overexpression in *Drosophila PG* could be partially rescued by feeding with 20E, and the animals could successfully complete the larval–pupal transition (Fig. 4*G* and *SI Appendix*, Fig. S13*A*). Collectively, our data indicate that *Hairy* expression in *Drosophila* PG represses ecdysone biosynthesis by negatively regulating the transcription of steroidogenic enzymes.

To determine the epistatic relationship between *Su(z)12* and *Hairy* in the PG, we surveyed the effect of *Hairy* knockdown in *Drosophila* larvae with PG-specific *Su(z)12* knockdown. Compared with only *Su(z)12* knockdown, the knockdown of both *Hairy* and *Su(z)12* in the PG increased the number of larvae undergoing the larval–pupal transition and ecdysone production (Fig. 4 *H* and

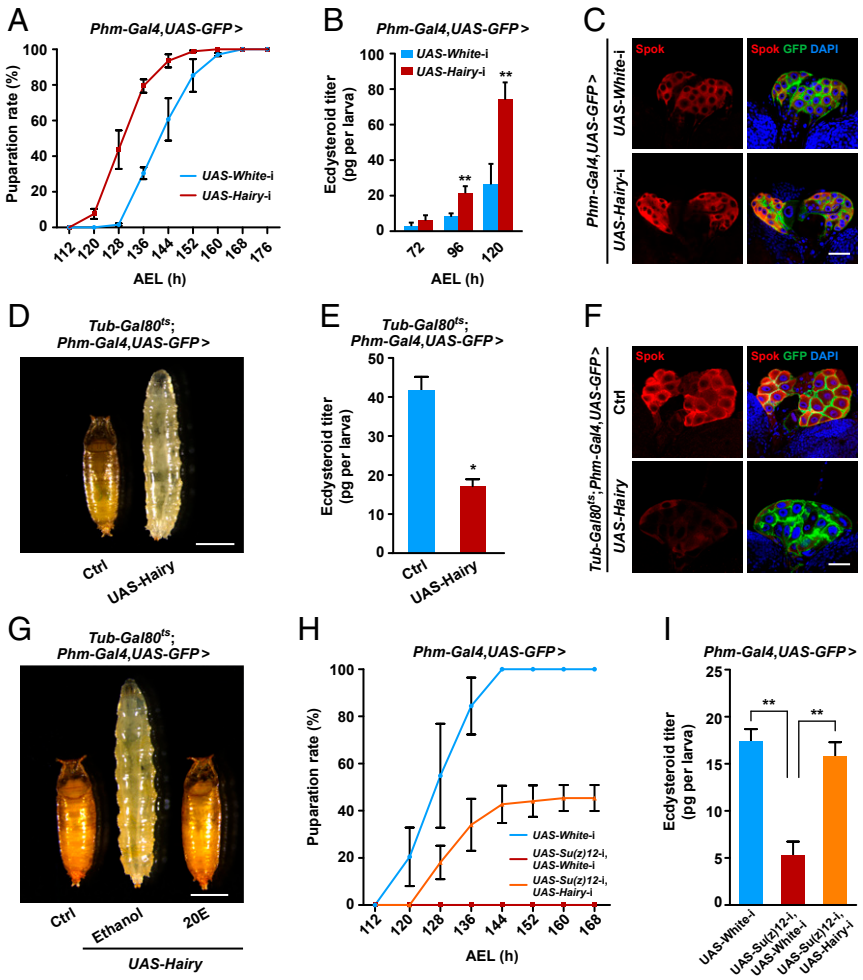

Fig. 4. Changes of *Hairy* expression in *Drosophila* PG affect the larval–pupal transition and impair both ecdysone production and the expression of steroidogenic enzymes. (*A*–*C*) PG-specific knockdown of *Drosophila Hairy* promoted the larval–pupal transition (*A*), increased ecdysone production (*B*), and up-regulated steroidogenic enzyme expression (*C*). The values are represented as the mean ± SE (error bars). (Scale bar, 20 μm.) (*D*–*F*) PG-specific overexpression of *Drosophila Hairy* led to developmental arrest at the third larval instar (*D*), decreased ecdysone production (*E*), and down-regulated steroidogenic enzyme expression (*F*). *Drosophila* larvae and the brain–RG complex were collected on the third day after shifting to 29 °C. (Scale bars, 1 mm, *D*, and 20 μm, *F*.) (*G*) The developmental arrest caused by *Hairy* overexpression in *Drosophila* PG could be partially rescued by feeding with 20E at 36 h after shifting to 29 °C. (Scale bar, 1 mm.) (*H* and *I*) The developmental defects (*H*) and decreased ecdysone production (*I*) caused by *Su(z)12* knockdown in *Drosophila* PG could be partially rescued by PG-specific *Hairy* knockdown. The Ctrl denotes a control by crossing *Phm-Gal4,UAS-GFP* with *w1118*. The values are represented as the mean ± SE (error bars). For the significance test, $*P < 0.05$ and $**P < 0.01$ versus the control.

I and *SI Appendix*, Fig. S13 *B*–*D*). At 144 h AEL, when the control larvae completed the larval–pupal transition, most *Su(z)12* knockdown animals remained fed, while approximately half of the animals with knockdown of both *Hairy* and *Su(z)12* entered pupariation (Fig. 4*H*). Taken together, the results indicate that the H3K27me3-mediated repression of *Hairy* transcription in the PG of insect larvae is required for the transcription of steroidogenic enzymes and ecdysone biosynthesis.

JH Represses PRC2-Mediated H3K27 Methylation in the PG of *Bombyx* and *Drosophila* Larvae. Previous studies have demonstrated that insect Hairy is induced by JH and regulates JH action (33–35). Given that the H3K27 methylation–mediated repression of *Hairy* transcription in the PG is required for ecdysone biosynthesis, we further investigated whether JH regulates PRC2-mediated H3K27 methylation. We first used the JH mimic (JHM) methoprene to treat ex vivo–cultured PG from *Bombyx* larvae on the sixth day of the last larval instar and found that JHM treatment reduced H3K27me3 levels in the PG compared with those obtained from the DMSO treatment as a control (Fig. 5*A* and *SI Appendix*, Fig. S14 *A* and *B*). An RT-qPCR analysis showed that mRNA expression of the *Su(z)12* gene was significantly down-regulated after JHM application (Fig. 5*B*). Similarly, JHM treatment of the ex vivo–cultured brain–RG complex from *Drosophila* larvae at 108 h AEL also decreased H3K27me3 levels and impaired *Su(z)12* transcription (Fig. 5 *C* and *D* and *SI Appendix*, Fig. S14*C*). Altogether, these data indicate that JH inhibits PRC2-mediated H3K27 methylation to maintain *Hairy* transcription in the PG of insect larvae.

Discussion

Insect development is cooperatively regulated by ecdysone and JH in opposite manners (2, 3, 5). Previous reports in *Drosophila* and *Bombyx* have uncovered JH-mediated transcriptional regulation of ecdysone biosynthesis in which JH directly acts on the PG to prevent precocious pupariation by repressing ecdysteroid biosynthesis (6, 7), and the JH signal transducer Kr-h1 directly inhibits the transcription of steroidogenic enzyme genes (6). Besides, increasing evidence have shown that the PRC2-catalyzed H3K27 methylation is involved in the development, differentiation, and proliferation in

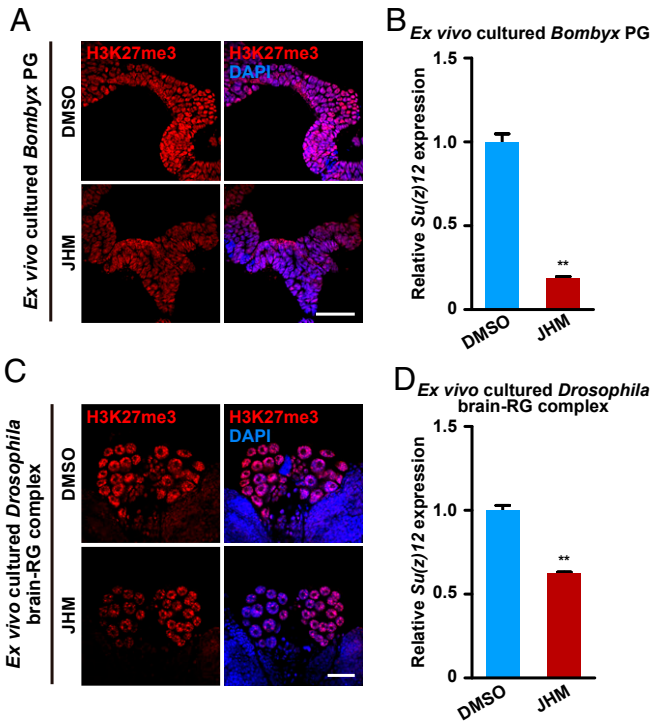

Fig. 5. JH represses H3K27 methylation in the PG. (*A* and *B*) The treatment of JHM methoprene reduced H3K27me3 levels and *Su(z)12* expression in ex vivo–cultured PG from *Bombyx* larvae on the sixth day of the last larval instar. (Scale bar, 200 μm.) (*C* and *D*) JHM treatment decreased H3K27me3 levels and *Su(z)12* expression in the ex vivo–cultured brain–RG complex containing the PG from *Drosophila* larvae at 108 h AEL. (Scale bar, 20 μm.) All experiments were conducted with three biological replicates. The values are represented as the mean ± SE (error bars). For the significance test, $^{**}P < 0.01$ versus the control.

eukaryotes by maintaining chromatin compaction at target genes and thereby leading to epigenetic transcriptional repression (18). In the present study, we demonstrate that H3K27 methylation targets another JH transducer gene *Hairy* in the PG of *Bombyx* and *Drosophila* larvae to regulate ecdysone biosynthesis and the larval–pupal transition (*SI Appendix*, Fig. S15). Briefly, high JH levels at the early stage of larval instar repress PRC2-mediated H3K27 methylation and thereby induce *Hairy* transcription and impair the transcription of steroidogenic enzymes as well as ecdysone biosynthesis in the PG. In contrast, increased H3K27me3 levels in the PG following low JH levels inhibit *Hairy* transcription and promote ecdysone biosynthesis. Taken together, these data suggest that JH repression of ecdysteroid biosynthesis and pupariation progression in insects involves transcriptional and epigenetic controls.

A striking finding of our study is that the PRC2-mediated H3K27 methylation in the PG during the larval period in insects regulates larva–pupal transition by positively modulating ecdysone biosynthesis. Previous studies in *Drosophila* revealed that the demethylation defect–induced increase in H3K27me3 levels at genes involved in ecdysone action during the pupal period delayed apoptosis and autophagy-mediated death of salivary glands (27). Notably, several other types of epigenetic markers have been also shown to modulate hormone action, including negative regulation of DNA methylation on ecdysone biosynthesis (6), microRNA inhibition of ecdysone production (40, 41), and positive regulation of histone acetylation on JH signaling (42, 43). Moreover, in addition to methylation, other posttranslational modifications of histones, including acetylation and ubiquitination, also direct different chromatin states and orchestrate gene expression (44). Therefore, although our data together with previous reports uncover an epigenetic regulatory network underlying the hormone control of insect development, it is worthy of further deciphering the relationship between H3K27 methylation and other known epigenetic regulations in insect hormone action.

We mechanistically deciphered that the JH-mediated repression of PRC2 activity in the PG during insect larval development maintains low H3K27me3 levels at the *Hairy* locus and promotes *Hairy* transcription, which in turn impairs ecdysone biosynthesis. Previous studies have reported that Hairy is a bHLH transcription factor and functions as a transcriptional repressor of genes associated with several developmental processes in *Drosophila*, including embryonic segmentation, neurogenesis, and sex determination (45–48). Intriguingly, another JH signal transducer Kr-h1 and nuclear receptor HR4 have been also characterized as repressors of both steroidogenic enzyme transcription and ecdysone production (6, 7, 49). However, the evidence in *Aedes aegypti* reveal that Hairy acts synergistically with Kr-h1 to mediate JH action (33–36). Collectively, it raised a possibility that Hairy may link with Kr-h1 and HR4 to regulate ecdysone production. Our preliminary investigation in *Drosophila* observed that PG-specific *Su(z)12* knockdown increased the transcription of the *HR4* gene, whereas *HR4* knockdown did not alter H3K27 methylation (*SI Appendix*, Fig. S16). These data, together with the fact that *Bombyx HR4* was excluded in H3K27me3 target collection from ChIP-seq data (Dataset S1), suggest that PRC2-mediated H3K27 methylation was indirectly involved in regulating *HR4* transcription in the PG. Whether and how Hairy couples with Kr-h1 and/or HR4 to participate in JH repression on ecdysone production remain to be determined.

Materials and Methods

Insect rearing, hormone treatment, RT-qPCR, immunostaining, Western blot, ecdysteroid measurement, ChIP-seq, ChIP-qPCR, luciferase reporter assay, and statistical analysis were performed, as described previously (6, 50). A detailed description of the materials and methods is provided in *SI Appendix*, *SI Materials and Methods*.

Data Availability. ChIP-seq raw data have been deposited in the National Center for Biotechnology Information Short Read Archive (accession no. PRJNA681675) (51). All other study data are included in the article and/or supporting information.

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