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20-Hydroxyecdysone (20E) Primary Response Gene *E75* Isoforms Mediate Steroidogenesis Autoregulation and Regulate Developmental Timing in *Bombyx*[*][S]

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The temporal control mechanisms that precisely control animal development remain largely elusive. The timing of major developmental transitions in insects, including molting and metamorphosis, is coordinated by the steroid hormone 20-hydroxyecdysone (20E). 20E involves feedback loops to maintain pulses of ecdysteroid biosynthesis leading to its upsurge, whereas the underpinning molecular mechanisms are not well understood. Using the silkworm *Bombyx mori* as a model, we demonstrated that *E75*, the 20E primary response gene, mediates a regulatory loop between ecdysteroid biosynthesis and 20E signaling. E75 isoforms A and C directly bind to retinoic acid receptor-related response elements in Halloween gene promoter regions to induce gene expression thus promoting ecdysteroid biosynthesis and developmental transition, whereas isoform B antagonizes the transcriptional activity of isoform A/C through physical interaction. As the expression of *E75* isoforms is differentially induced by 20E, the E75-mediated regulatory loop represents a fine autoregulation of steroidogenesis, which contributes to the precise control of developmental timing.

Animals undergo developmental transitions from the embryo to juvenile to adulthood, and these processes are determined by steroid hormones and their corresponding nuclear receptors (NRs).[2] In insects, 20-hydroxyecdysone (20E; ecdysone is the immediate precursor of 20E; 20E and ecdysone are the main ecdysteroids) is the actual steroid hormone. The ecdysone receptor (EcR) and its partner molecule, Ultraspiracle (USP), form the functional NR complex of 20E. In conjunction with EcR-USP, 20E activates a small set of early response genes encoding several transcription factors that further activate a large set of downstream late response genes. Pulses of 20E signals initiate major developmental transitions in insects, including egg hatching, larval-larval molting, and larval-pupal-adult metamorphosis (1, 2).

NRs form a large and conserved superfamily of ligand-activated transcription factors that are essential for growth, development, reproduction, homeostasis, and metabolism. NRs are defined by the presence of a highly conserved DNA binding domain (DBD) and a less conserved ligand binding domain (3, 4). There are 18–19 NRs in insects, including the fruit fly, *Drosophila melanogaster*, and the silkworm, *Bombyx mori* (3, 5, 6). Apart from the EcR, ligand was only identified for another insect NR, ecdysone-induced protein 75B (E75). *E75* is a crucial 20E response gene that affects ecdysteroid titer. E75 binds to heme, which responds to gases NO and CO (7–12). The E75 orthologs in mammals are Rev-erb α (NR1D1) and Rev-erb β (NR1D2), and NR1D2 binds to heme, responds to NO, and regulates circadian rhythm (13–15).

In *Drosophila*, the *E75* locus encodes four *E75* mRNA isoforms, *E75A*, *E75B*, *E75C*, and *E75D*, which are generated by differential promoter usage and alternative splicing of 5′ exons. The DBD of E75A/C possesses two C4 zinc fingers; E75B is incomplete and contains only one zinc finger, whereas E75D lacks a DBD. 20E-EcR-USP rapidly and abundantly induces the expression of *E75A* and *E75B* by binding to the 20E response elements present in the promoter regions. In contrast, the 20E induction of *E75C* expression is slow and weak (9, 16). Germ

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[S] This article contains supplemental Figs. S1–S5.

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[2] The abbreviations used are: NR, nuclear receptor; 20E, 20-hydroxyecdysone; EcR, ecdysone receptor; RORE, retinoic acid receptor-related response element; USP, Ultraspiracle; DBD, DNA binding domain; ACN, acetonitrile; IW, initiation of wandering; BmNPV, *B. mori* nucleopolyhedrosis baculovirus; dsRNA, double-stranded RNA; qPCR, quantitative PCR; rpHPLC, reverse-phase HPLC; EIA, enzyme immunoassay.

line clones of *E75*-null mutants missing all three isoforms lead to arrest during mid-oogenesis (17). Isoform-specific *E75* null mutants exhibit different phenotypes; *E75A* mutants show a reduced ecdysteroid titer leading to developmental retardation and molting defects; *E75B* mutants can survive and exhibit normal reproductive performance; and *E75C* mutants die within a few days after eclosion (9). E75 might regulate 20E signals through interaction with another 20E response gene *HR3*, which encodes another important insect NR. HR3 controls the termination of the 20E signal pulse, which triggers the larval-prepupal transition by both inhibiting 20E-EcR-USP transactivation by interacting with EcR and blocking ecdysone biosynthesis by down-regulating the Halloween family of cytochrome P450 genes (Halloween genes). HR3 also induces the expression of *βftz-F1*, which acts as a competent factor for EcR-USP to respond to the subsequent 20E signal pulse during the prepupal-pupal transition. Importantly, E75 acts as a transcriptional repressor for HR3 in relieving HR3 inhibition on 20E signaling and HR3 induction on *βftz-F1* expression. E75 inhibits the transactivation ability of HR3 through physical interaction and competing for binding to the retinoic acid receptor-related receptor response elements (ROREs). Therefore, the 20E-induced transcriptional cascade, including EcR-USP, E75, HR3, and βftz-F1, governs the larval-prepupal-pupal transition. In addition, because NO and CO are able to reverse the ability of E75 to interfere with HR3, the function of E75 is modulated by gas availability (10–12, 18–21).

Early studies found that E75B interferes with HR3 induction of *βftz-F1* expression (18), and later studies revealed that at least E75A has the same function (11), indicating that E75 isoforms play similar roles in HR3 regulation. However, in female adults, E75A induces apoptosis in the egg chamber at stages 8 and 9, whereas E75B prevents E75A function and thus allows egg development, indicative of opposite roles in regulating female reproduction (22). Similarly, E75 isoforms also play distinct roles in regulating female reproduction in the mosquito, *Aedes aegypti* (23). Given that both E75A and E75B have similar effects on HR3, HR3 clearly cannot account for the opposite functions of the E75 isoforms, suggesting that E75 isoforms may employ novel mechanisms to differentially regulate insect development.

Bombyx E75 processes at least three isoforms, *E75A*, *E75B*, and *E75C*, showing similar gene organization and 20E response to *Drosophila E75* (24, 25). Likewise, *Bombyx* E75A/C interacts with HR3 and represses its transactivation activity by physical interaction and competing for ROREs (26). We reasoned that *Bombyx* could be a good model to solve the E75 isoform-specific mechanism, because this insect species has a comparatively longer life cycle for phenotypic observations and can be genetically modified for functional analyses (27). A molecular dissection of E75 isoforms in *Bombyx* found that, in addition to acting as transcriptional repressors of HR3, E75 isoforms also regulate ecdysteroid biosynthesis by directly controlling Halloween gene expression. Mechanistically, E75A/C functions as a transcriptional factor to directly induce Halloween gene expression, whereas E75B antagonizes the transactivation ability of E75A/C. Given that the expression of E75 isoforms is differentially induced by 20E, our study revealed an E75-mediated regulatory loop that contributes to steroidogenesis autoregulation and thus developmental timing. Regarding the ultimate regulation of ecdysteroid biosynthesis, E75 first functions directly and then acts through inhibition of HR3.

Results

E75 RNAi Disrupts 20E Signaling and 20E-induced Metamorphosis—We have previously demonstrated that *E75* isoforms display stage- and tissue-specific responses to 20E (25). To determine the function of E75 during larval-pupal metamorphosis, expression of all three *E75* isoforms was reduced by RNAi (*E75* RNAi) at the initiation of the wandering stage (IW). *E75* RNAi caused lethal phenotypes, with ~60 and 10% lethality during the prepupal and pupal stages, respectively. Some *E75* RNAi larvae died during the wandering stage, and others failed to form normal pupae and died as larval-pupal intermediates, whereas others were arrested during the pupal stage or immediately after adult emergence (Fig. 1, *A*–*A*‴).

Importantly, *E75* RNAi inhibited fat body remodeling, which is controlled mainly by the 20E-triggered transcriptional cascade during larval-pupal metamorphosis (28–31). Twenty four hours after injection with *E75* double-stranded RNA (dsRNA) (supplemental Fig. S1, *A* and *B*′), LysoTracker Red staining, the number and size of autophagosomes and the ATG8 protein levels decreased significantly, suggesting that the 20E-induced fat body autophagy is affected by *E75* RNAi (Fig. 1, *B*–*B*″). Meanwhile, labeling with Hoechst 33342 and propidium iodide, TUNEL staining, and measurement of caspase 3 activity revealed significant reductions in 20E-induced fat body apoptosis by *E75* RNAi (Fig. 1, *C*–*C*″). In addition, the 20E-induced fat body cell dissociation that occurred 24 h after pupation in the *EGFP* RNAi control pupae was significantly prevented in the *E75* RNAi pupae (Fig. 1*D*).

The effects of *E75* RNAi on fat body remodeling suggest that E75 is required for maintaining 20E signaling to promote larval-pupal metamorphosis. The expression levels of several key genes in the 20E-triggered transcriptional cascade were determined by quantitative real time PCR (qPCR) using the total RNA isolated from the fat body collected 24 h after *E75* dsRNA injection. The mRNA levels of all the 20E-response genes decreased by 60–90% compared with their levels in the control larvae (Fig. 1*E*). Moreover, Western blottings using EcR-B1, USP, and Br-C antibodies revealed a decrease in their protein levels in the *E75* RNAi larvae (Fig. 1*E*′), indicating that *E75* RNAi disrupts the 20E-triggered transcriptional cascade in the fat body during larval-pupal metamorphosis. Overall, *E75* RNAi disrupted 20E signaling, prevented fat body remodeling, and caused lethality during metamorphosis.

E75 RNAi Down-regulates the Halloween Genes and Decreases Ecdysteroid Biosynthesis—Several genes in the 20E-triggered transcriptional cascade, including *EcR*, *Br-C*, *E75*, *HR3*, and *βftz-F1*, regulate ecdysteroid titers in *Drosophila* (9, 21, 32, 33). We recently identified the role of *E93* in maintaining ecdysteroid titers in *Bombyx* (31). As measured by enzyme immunoassay (EIA) 24 h after dsRNA treatment, ecdysteroid titers significantly decreased in *E75* RNAi larvae (Fig. 2*A*), suggesting that E75 is required for maintaining ecdysteroid titers in *Bombyx*.

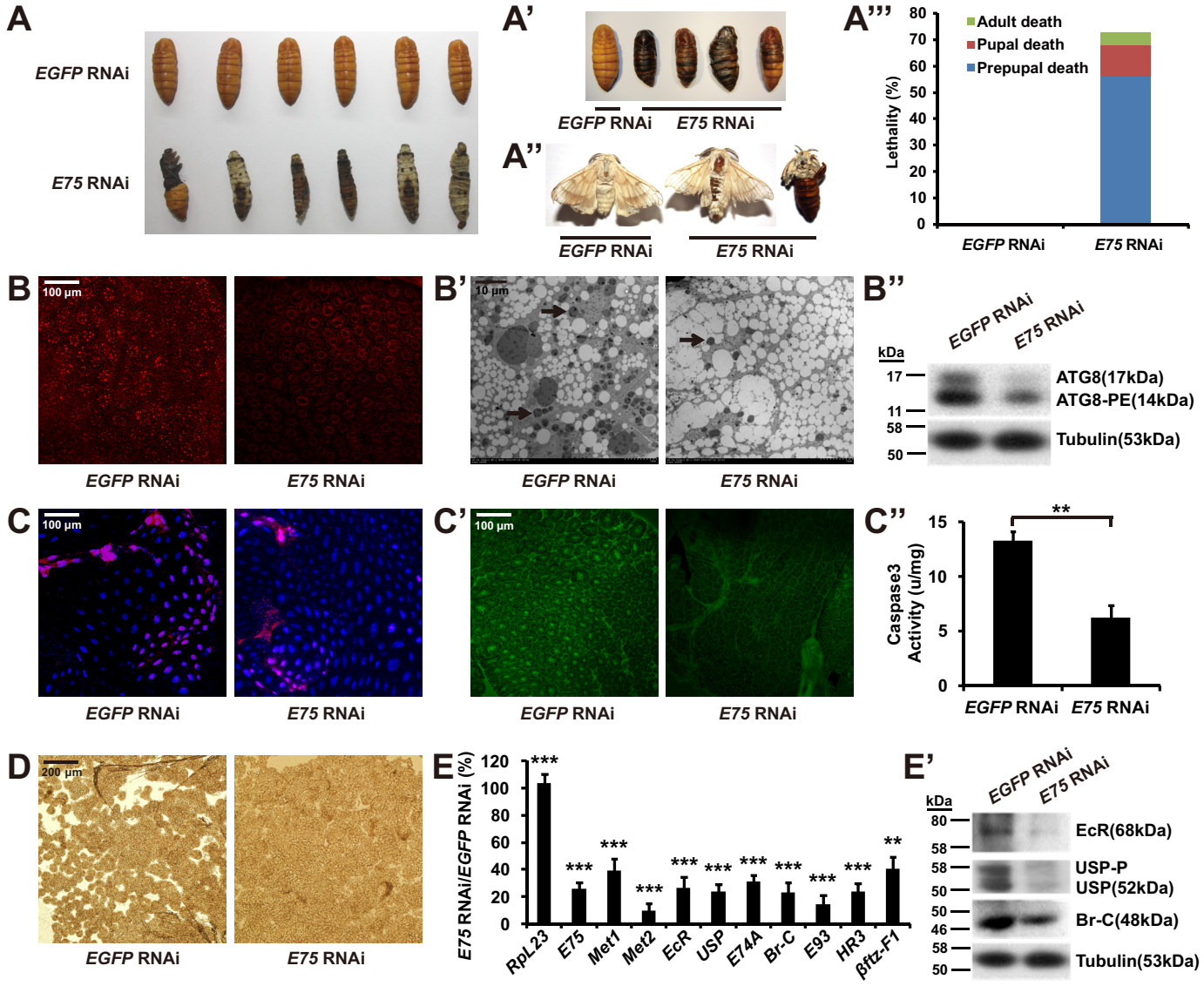

FIGURE 1. **E75 RNAi disrupts 20E signaling and 20E-induced metamorphosis.** *E75* dsRNA (30 μg per larva) was injected into each *Bombyx* larva at the initiation of the wandering stage. *EGFP* dsRNA was used as a control. *A* and *A‴*, *E75* dsRNA-treated silkworms died during the prepupal (*A*), pupal (*A′*), and adult (*A″*) stages. The chart (*A‴*) shows the quantification of the lethality during the prepupal, pupal, and adult stages. *B* and *B″*, LysoTracker Red staining (*red,* ×40) (*B*); transmission electron microscopy analysis (×7500; *black arrow* denotes an autolysosome) (*B′*), and Western blotting of ATG8 (*B″*) in the fat body 24 h after dsRNA treatment. *C* and *C″*, Hoechst 33342 (*blue*) and propidium iodide (*red*) staining (×40) (*C*); TUNEL labeling (*green*, ×40) (*C′*), and caspase 3 activity (*C″*) in the fat body 24 h after dsRNA treatment. *D*, comparison of fat body cell dissociation at 24 h after pupation. *E* and *E′*, qPCR analysis of 20E-response genes (*E*) and Western blotting analysis of the protein levels of some 20E-response genes (*E′*) in the fat body at 24 h after dsRNA treatment.

The prothoracic glands produce and secrete ecdysone; once released into the hemolymph, ecdysone is converted to 20E in the peripheral tissues, such as the fat body and midgut (34). Ecdysone and 20E in the mixture of hemolymph ecdysteroids were separated and individually collected using reverse-phase high performance lipid chromatography (rpHPLC) and then measured by EIA. As expected, both titers of ecdysone and 20E decreased in *E75* RNAi larvae; moreover, the ratio between 20E and ecdysone was further decreased in *E75* RNAi larvae (Fig. 2, *B*–*B″*).

In *Bombyx*, the Halloween genes, *spook* (*spo*), *phantom* (*phm*), *disembodied* (*dib*), and *shadow* (*sad*), mediate the sequential steps of ecdysone biosynthesis in the prothoracic glands, whereas *shade* (*shd*) catalyzes the conversion from ecdysone to 20E in the fat body and other peripheral tissues (35, 36). The mRNA levels of *spo*, *phm*, *dib*, and *sad* decreased by more than 90% in the prothoracic glands isolated from *E75* RNAi larvae (Fig. 2*C*). The prothoracic glands were dissected out from the *E75* RNAi larvae and cultured *in vitro*, and the ecdysone released into the medium was measured by EIA. Importantly, the ratio of ecdysone release by the cultured prothoracic glands decreased by about half in *E75* RNAi larvae (Fig. 2*C′*). Overall, the prothoracic glands of *E75* RNAi larvae exhibited normal morphology without apparent autophagy, apoptosis, and cell dissociation (supplemental Fig. S2, *A* and *C*). Nevertheless, a large number of mitochondria, which are essential for hormone production in endocrine organ cells, were misshaped in the prothoracic gland cells from the *E75* RNAi larvae, supporting the reduced ecdysone production (Fig. 2*C″*). Meanwhile, the mRNA levels of *shd* decreased by 80% in the fat body

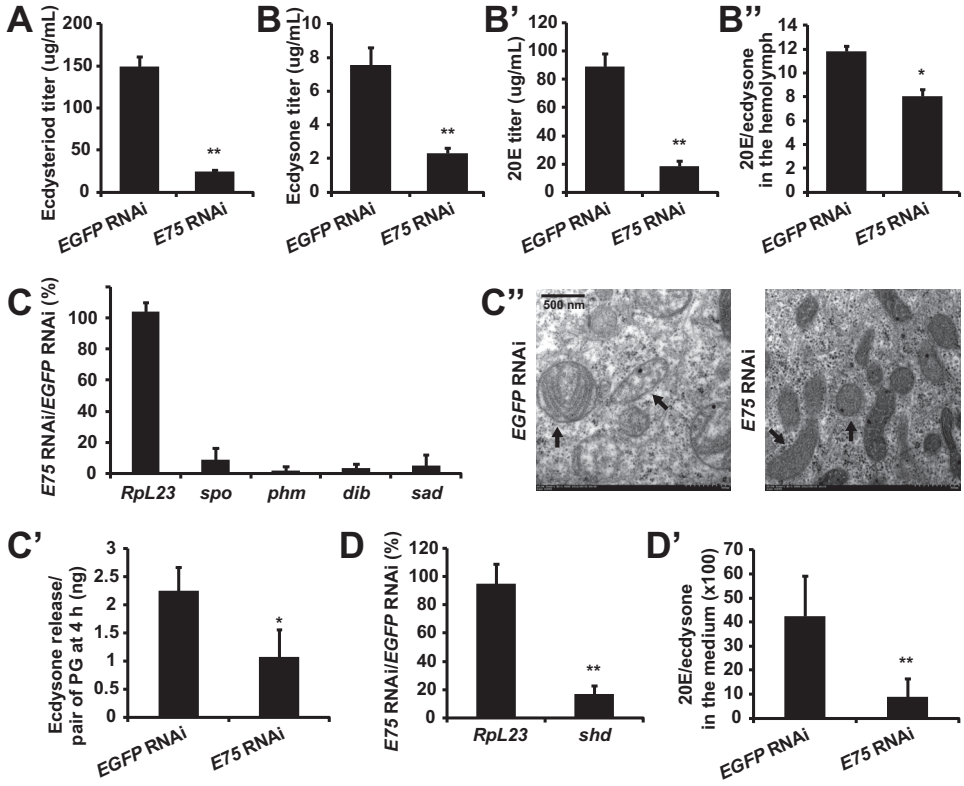

FIGURE 2. **E75 RNAi down-regulates the Halloween genes and decreases ecdysone biosynthesis and the conversion of ecdysone to 20E.** *E75* dsRNA (30 μg per larva) was injected into each *Bombyx* larvae at the initiation of the wandering stage. *EGFP* dsRNA (30 μg per larva) was used as a control. *A*, comparison of ecdysteroid levels at 24 h after dsRNA treatment. *B–B″*, comparisons of the levels of ecdysone (*B*) and 20E (*B′*) as well as the ratio between 20E and ecdysone (*B″*) in the hemolymph at 24 h after dsRNA treatment. *C–C″*, qPCR analysis of four Halloween genes (*spo*, *phm*, *dib*, and *sad*) in prothoracic glands (*C*), transmission electron microscopy analysis of the mitochondria (8000×; the *arrow* denotes mitochondria) in prothoracic glands (*C′*), and a comparison of ecdysone release in the prothoracic glands at 24 h after dsRNA treatment. *D* and *D′*, qPCR analysis of the Halloween gene *shd* (*D*) and a comparison of the conversion of ecdysone to 20E (*D′*) in the fat body at 24 h after dsRNA treatment.

from the *E75* RNAi larvae (Fig. 2*D*). The fat body tissues were dissected and cultured *in vitro* with the addition of ecdysone in the medium, and ecdysone and the newly converted 20E in the medium were separated by rpHPLC and measured by EIA. The conversion from ecdysone to 20E also decreased by 80% in the fat body dissected from the *E75* RNAi larvae (Fig. 2*D′*). Taken together, these data demonstrated that *E75* RNAi down-regulates Halloween genes that are responsible for ecdysone biosynthesis in the prothoracic glands and the conversion from ecdysone to 20E in the fat body, resulting in the disruption of ecdysteroid biosynthesis and 20E-induced metamorphosis.

Overexpression of E75A/C Up-regulates Halloween Genes, Promotes 20E Signaling, and Accelerates Metamorphosis—Initial experiments using RNAi to reduce the expression of each *E75* isoform showed variable results, mostly because their AF-1 domains are too short to generate reliable isoform-specific dsRNAs. We generated an ecdysteroid UDP-glucosyltransferase (*egt*) mutant of *B. mori* nucleopolyhedrosis baculovirus (BmNPV) to overexpress each *E75* isoform on day 2 of the fifth instar (L5D2). Five and a half days after BmNPV infection, only 30% of the *EGFP*-overexpressed larvae began wandering, whereas 80 and 70% of the *E75A*- and *E75C*-overexpressed larvae entered the wandering stage with reduced body sizes, respectively, although the wandering behavior and the body size of the *E75B*-overexpressed larvae were slightly prevented compared with the control larvae (Fig. 3, *A* and *A′* and supplemental Fig. S1, *C* and *D‴*). Moreover, 72 h after BmNPV infection, both titers of ecdysone and 20E increased in the *E75A/C*-overexpressed larvae, but they slightly decreased in the *E75B*-overexpressed larvae (Fig. 3, *B–B″*).

Seventy two hours after BmNPV infection, we further examined the effects of each E75 isoform on the prothoracic glands and fat body (supplemental Fig. S1, *C* and *D‴*). The mRNA levels of *spo*, *phm*, *dib*, and *sad* increased by 4–7- and 3–5-fold in the prothoracic glands of the *E75A*- and *E75C*-overexpressed larvae, respectively; however, they decreased by 20–40% in the *E75B*-overexpressed larvae (Fig. 3, *C–C′*). The amount of ecdysone released by the cultured prothoracic glands increased by 200 and 150% in the *E75A*- and *E75C*-overexpressed larvae, respectively, but they slightly decreased in the *E75B*-overexpressed larvae (Fig. 3*D*). Meanwhile, the mRNA levels of *shd* increased by 3-fold in the fat body from the *E75A/C*-overexpressed larvae, but they slightly decreased in the *E75B*-overexpressed larvae (Fig. 3*E*). Similarly, the conversion from ecdysone to 20E increased in the fat body from the *E75A/C*-overexpressed larvae, but they slightly decreased in the *E75B*-overexpressed larvae (Fig. 3*F*). In conclusion, overexpression of *E75A/C* up-regulates Halloween genes, promotes ecdysteroid biosynthesis, and accelerates metamorphosis, whereas *E75B* overexpression might have opposing effects.

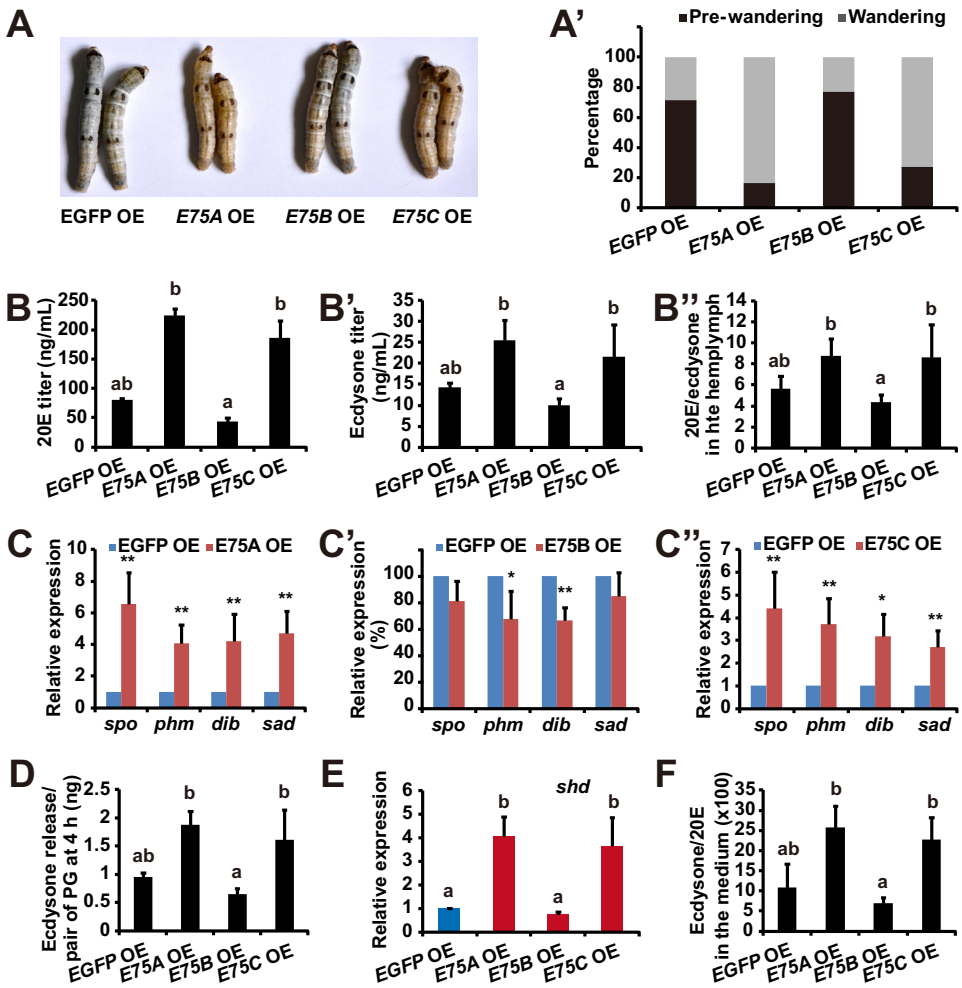

FIGURE 3. **Overexpression of *E75A/C* up-regulates Halloween genes, promotes 20E signaling, and accelerates metamorphosis.** P2 BmNPV *egt* mutant expressing *E75A/B/C* (5 μl; ~10^5 pfu) was injected into each *Bombyx* larva on day 2 of the fifth instar. BmNPV expressing *EGFP* was used as a control. *A* and *A′*, changes in wandering behavior and body size at ~5.5 days after injection of BmNPV *E75A/B/C* virus (*A*). The *chart* (*A′*) shows the quantification of the wandering behavior observed. *B–B″*, comparisons of levels of ecdysone (*B*) and 20E (*B′*) as well as the ratio between 20E and ecdysone (*B″*) in the hemolymph at 72 h after injection of BmNPV *E75A/B/C* virus. *C* and *D*, qPCR analysis of four Halloween genes (*spo*, *phm*, *dib*, and *sad*) (*C–C″*) and a comparison of ecdysone release (*D*) in the prothoracic glands at 72 h after injection of BmNPV *E75A/B/C* virus. *E* and *F*, qPCR analysis of the Halloween gene *shd* (*E*) and a comparison of the conversion from ecdysone to 20E (*F*) in the fat body at 72 h after injection of BmNPV *E75A/B/C* virus.

E75A/C, but Not E75B, Binds to ROREs and Directly Induces Halloween Gene Expression—Because E75 binds to ROREs to antagonize the transactivation ability of HR3, we hypothesized that E75 might also bind to ROREs and thus directly induce Halloween gene expression. Using a dual-luciferase assay system established in heterologous human HEK 293 cells, we investigated whether the three E75 isoforms can directly bind the promoter of the five Halloween genes, including *spo*, *phm*, *dib*, *sad*, and *shd*. The ~2.5-kb promoter region of each Halloween gene was cloned into the pGL3 vector. Upon *E75A/C* overexpression, all five ~2.5-kb promoter regions supported a 2.5–4-fold increase in luciferase activity, whereas *E75B* overexpression had no effect (Fig. 4, *A–E*). In BmN cells, the luciferase activities of all five ~2.5-kb promoter regions increased 1.5–3-fold upon *E75A/C* overexpression. Interestingly, *E75B* overexpression slightly reduced the luciferase activities (Fig. 4, *F–J*), resembling the effects of *E75B* overexpression *in vivo* (Fig. 3, *C–C″* and *E*).

There are 2, 1, 3, 2, and 4 potential RORE in the ~2.5-kb promoter regions of *spo*, *phm*, *dib*, *sad*, and *shd*, respectively (supplemental Fig. S3). We then performed chromatin immunoprecipitation (ChIP) in Bm-N cells to examine how the three E75 isoforms bind to RORE. The binding of E75 isoforms to DNA was detected using the V5 antibody and cross-linked chromatin isolated from Bm-N cells that were transfected with the *E75A/B/C-V5* expression plasmids. As measured by qPCR, the V5 antibody increased the precipitation of 13 RORE (except one in *shd*) when *E75A* was overexpressed, 12 RORE (except one in *sad* and the other in *shd*) when *E75C* was overexpressed, but no RORE when *E75B* was overexpressed (Fig. 4, *K–O*).

All the responsive RORE in the ~2.5-kb promoter regions of each Halloween gene were deleted, and the mutated ~2.5-kb promoter regions of all the five Halloween genes were cloned into the pGL3 vector. *E75A/C* overexpression did not increase luciferase activity for any of the mutated constructs (Fig. 4, *P–T*). Together, the dual-luciferase assays and ChIP-qPCR data revealed that E75A/C, but not E75B, binds to RORE in the promoter regions of all five Halloween genes and directly induces gene expression.

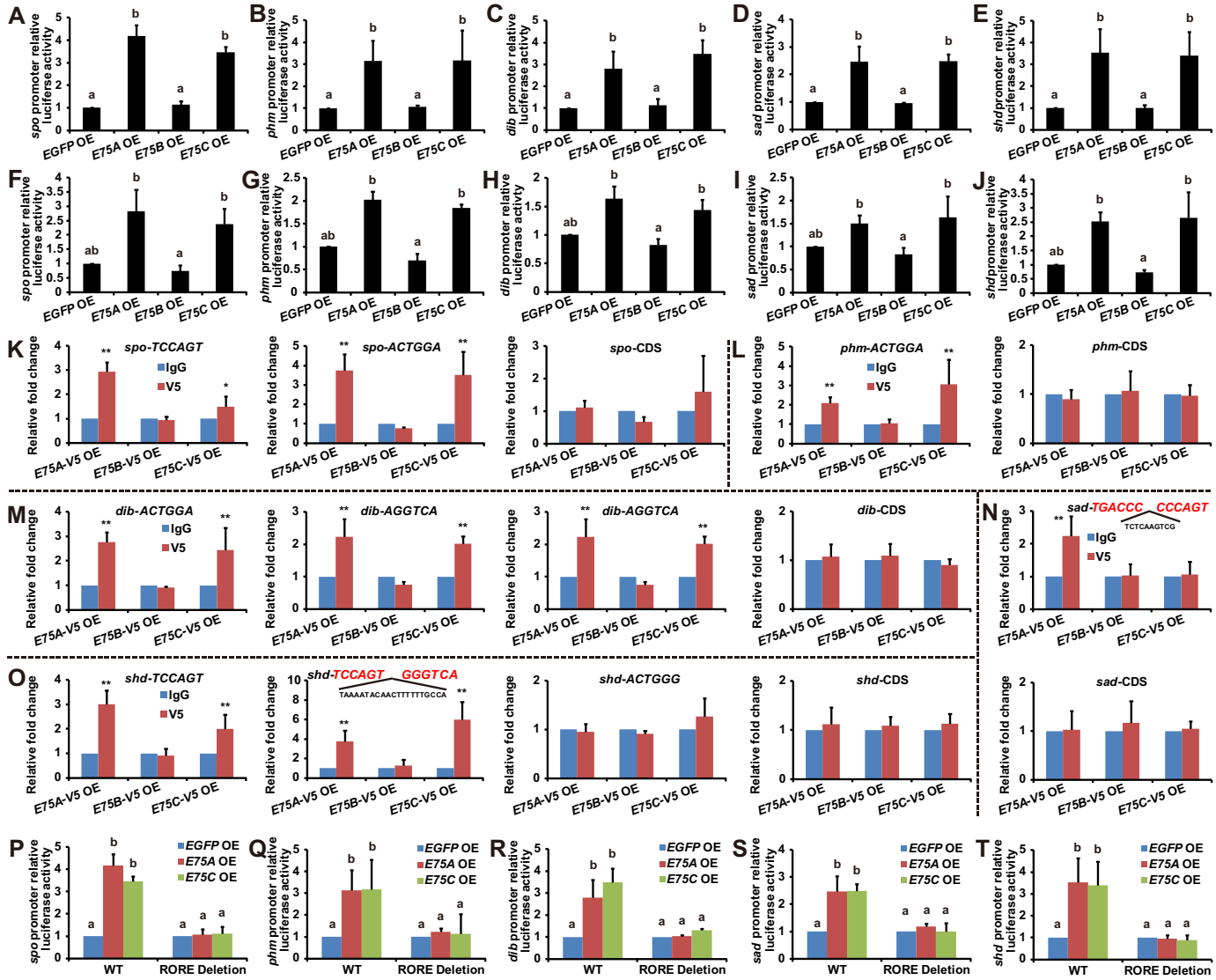

FIGURE 4. **E75A/C, but not E75B, bind to ROREs and induce Halloween gene expression.** *A–E,* HEK 293 cells were co-transfected with the *E75A/B/C* (*EGFP* as a control) expression construct, the pGL3 basic plasmids containing ~2.5-kb promoter regions of each Halloween gene (*spo* (*A*), *phm* (*B*), *dib* (*C*), *sad* (*D*), and *shd* (*E*)), the *hsp70* basal promoter regulating the expression of firefly luciferase (*Fluc*), and a reference reporter plasmid carrying *Renilla* luciferase (*Rluc*). After 48 h of transfection, the dual-luciferase assays were performed. The luciferase activity fold change is defined as the relative luciferase activity induced by *E75A/B/C* overexpression compared with *EGFP* overexpression. *F–J,* BmN cells were co-transfected with the *E75A/B/C* (*EGFP* as a control) expression construct, the pGL3 basic plasmids containing ~2.5-kb promoter regions of each Halloween gene (*spo* (*F*), *phm* (*G*), *dib* (*H*), *sad* (*I*), and *shd* (*J*)), and the *hsp70* basal promoter regulating the expression of firefly luciferase (*Fluc*), and a reference reporter plasmid carrying *Renilla* luciferase (*Rluc*). The dual-luciferase assays were performed as in *A–E*. *K–O,* ChIP assays of E75A/B/C-V5 binding to the ~2.5-kb promoter regions of Halloween gene promoters. Bm-N cells were transfected with *E75A/B/C-V5* expression plasmid for 48 h and immunoprecipitated with IgG or antibodies against V5. The precipitated DNA and input were analyzed by qPCR to detect the binding between E75A/B/C-V5 and ROREs in the promoter regions and CDS regions of the five Halloween genes. The results of qPCR analyses are presented as E75A/B/C-V5 compared with IgG. *P–T,* all the ROREs in the ~2.5-kb promoter regions of each Halloween gene were deleted, and the mutated ~2.5-kb promoter regions of all the five Halloween genes were cloned into the pGL3 vector. The dual-luciferase assays were performed as in *A–E*.

E75B Antagonizes the Transactivation Ability of E75A/C—The above overexpression results raise the possibility that E75B antagonizes the transactivation ability of E75A/C. To test this hypothesis, *E75A* or *E75C* and *E75B* were co-transfected into HEK 293 cells. The effect of the expressed proteins on *spo* and *shd* promoter activities was determined. As shown above (Fig. 4, *A–E*), *E75A/C* overexpression, but not *E75B* overexpression, showed significant increases in the luciferase activity. Importantly, co-transfection of *E75B* antagonized the transactivation ability of *E75A/C* in a dose-dependent manner, whereas *E75A* and *E75C* did not affect each other (Fig. 5, *A–B′*). Similar results were obtained in BmN cells (Fig. 5, *C–D′*).

To further verify the hypothesis *in vivo*, equal amounts of two BmNPVs of *EGFP*, *E75A*, *E75B*, or *E75C* were co-infected to L5D2 larvae. Five days after BmNPV infection, *E75A/C*-, but not *E75B*-, overexpressed larvae showed precocious wandering behavior and reduced body size compared with the *EGFP*-overexpressed control larvae (Fig. 5*E*). Importantly, co-infection with *E75B* nearly blocked the ability of *E75A/C* to reduce body size (Fig. 5*E′*). Seventy two h after BmNPV infection, ecdysteroid titers significantly increased in the *E75A/C*-overexpressed larvae, and this increase was blocked by co-infection with *E75B* (Fig. 5*F*), suggesting that E75B antagonizes the transactivation ability of E75A/C.

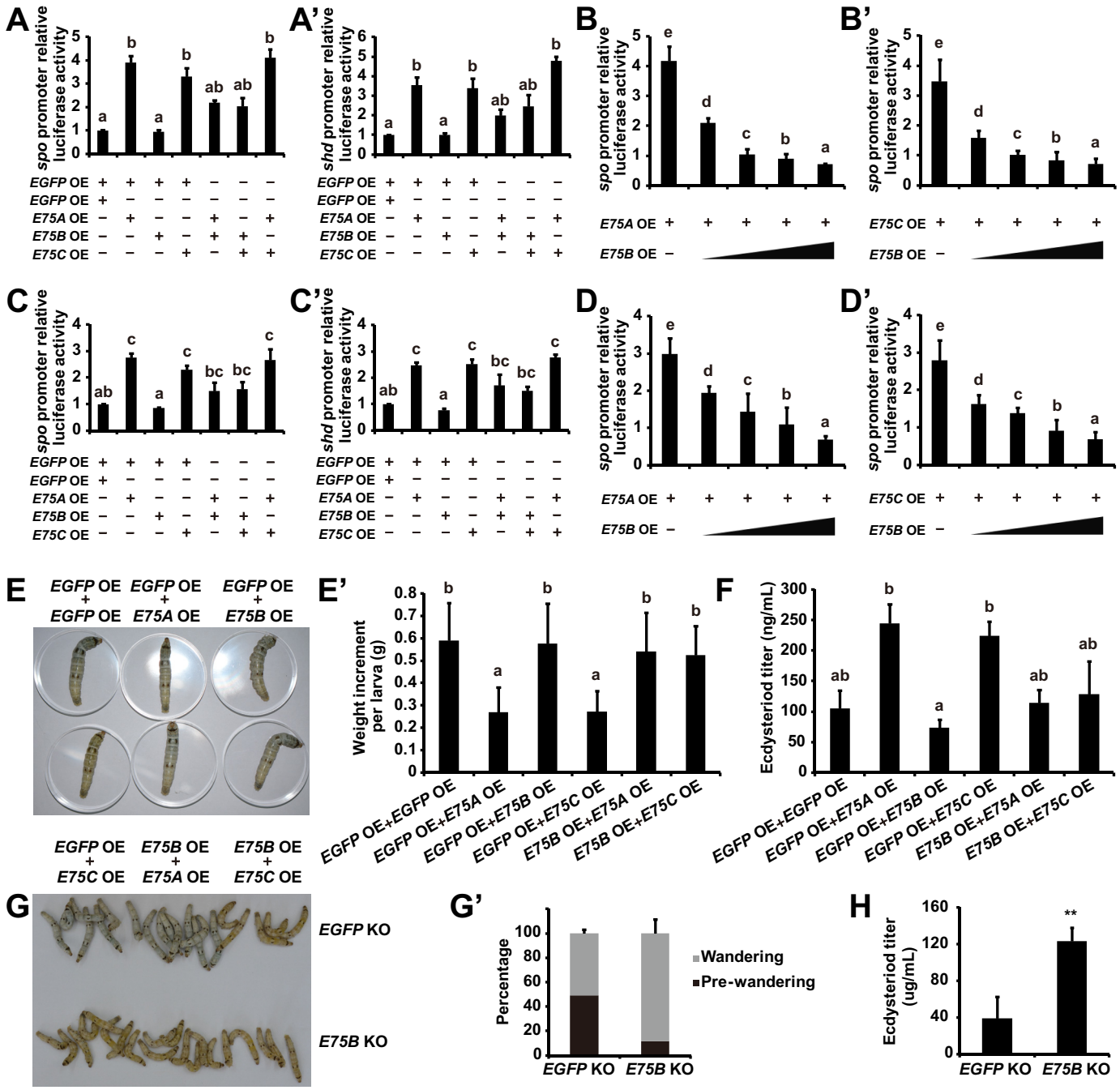

FIGURE 5. **E75B antagonizes the transactivation ability of E75A/C.** *A* and *A'*, HEK 293 cells were co-transfected with equal amounts of two expression constructs (*EGFP*, *E75A*, *E75B*, or *E75C*), the pGL3 basic plasmids containing ~2.5-kb promoter regions of *spo* (*A*) or *shd* (*A'*), and the *hsp70* basal promoter regulating the expression of firefly luciferase (*Fluc*), and a reference reporter plasmid carrying *Renilla* luciferase (*Rluc*). After 48 h of transfection, the dual-luciferase assays were performed. Luciferase activity fold change is defined as the relative luciferase activity compared with *EGFP*. *B* and *B'*, dual-luciferase assays in HEK 293 cells were performed as described in *A* and *A'*. Three expression constructs were used: *E75A/C*, a gradient amount of *E75B*, and *EGFP*; the amount of *E75A/C* equals *E75B* and *EGFP*. The pGL3 basic plasmid containing ~2.5-kb promoter regions of *spo* and the *hsp70* basal promoter regulating the expression of firefly luciferase were used. *C* and *C'*, BmN cells were co-transfected with equal amounts of two expression constructs (*EGFP*, *E75A*, *E75B*, or *E75C*), the pGL3 basic plasmids containing ~2.5-kb promoter regions of *spo* (*C*) or *shd* (*C'*), and the *hsp70* basal promoter regulating the expression of firefly luciferase (*Fluc*), and a reference reporter plasmid carrying *Renilla* luciferase (*Rluc*). The dual-luciferase assays were performed as in *A* and *A'*. *D* and *D'*, dual-luciferase assays in BmN cells were performed as described in *C* and *C'*. Three expression constructs were used: *E75A/C*, a gradient amount of *E75B*, and *EGFP*; the amount of *E75A/C* equals *E75B* and *EGFP*. The pGL3 basic plasmid containing ~2.5-kb promoter regions of *spo* and the *hsp70* basal promoter regulating the expression of firefly luciferase were used. *E* and *F*, changes in wandering behavior and body size at ~5 days after injection of BmNPV expressing *EGFP*, *E75A*, *E75B*, and *E75C* (*E*). The chart (*E'*) shows the quantification of the body size. A comparison of ecdysteroid titer at 72 h after injection of BmNPV expressing *EGFP*, *E75A*, *E75B*, and *E75C* (*F*). *G* and *H*, changes in wandering behavior after CRISPR/Cas9-mediated knock-out of *E75B* (*G*). The chart (*G'*) shows the quantification of the wandering behavior in *G*. A comparison of ecdysteroid titers at 24 h after the initiation of the wandering stage (*H*).

CRISPR/Cas9-mediated genome editing is becoming a powerful tool for functional studies in *Bombyx* (37, 38). Because RNAi was not able to sufficiently and specifically reduce *E75B* expression, we performed CRISPR/Cas9-mediated knock-out of *E75B*. Interestingly, all of the *E75B*-knock-out larvae successfully survived to adults but showed accelerated wandering

behavior and elevated ecdysteroid titers (Fig. 5, *G* and *H*, and supplemental Fig. S4). Overall, the *E75B*-knock-out larvae underwent phenotypic changes similar to those of the *E75A/C*-overexpressed larvae. Both *in vitro* and *in vivo* experimental data revealed that E75B antagonizes the transactivation ability of E75A/C for regulating Halloween gene expression, ecdysteroid biosynthesis, and metamorphosis.

Incomplete DBD in E75B Mediates Physical Interactions and Thus the Opposing Actions between E75B and E75A/C—Finally, we investigated whether E75B antagonizes the transactivation ability of E75A/C through protein-protein interactions. Two constructs of *E75A*, *E75B*, or *E75C*, the C termini of which were fused to different tags, were co-transfected in HEK 293 cells. Immunocytochemistry was performed to examine their possible protein-protein interactions. When *E75A* and/or *E75C* were co-transfected, they evenly localized in the nuclei (Fig. 6*A*). By contrast, when *E75B* was co-transfected with *E75A*, *E75B*, or *E75C*, the two proteins frequently co-localized at some aggregating chromatin spots (Fig. 6*B*). Similar results were obtained in BmN cells (Fig. 6, *C* and *D*). Both data in HEK 293 and BmN cell lines suggested that E75B might associate with all three E75 isoforms.

E75A, E75B, and E75C contain different AF-1 domains and DBDs (25). To identify the actual E75B domain(s) that are responsible for its association with all three E75 isoforms, we generated three *E75* mutant constructs: *coE75A/C* that shares the complete DBD of *E75A/C* and the common C terminus, *coE75A/B/C* that shares the incomplete DBD of *E75A/B/C* and the common C terminus, and *E75noN* that only retains the common C terminus (Fig. 6*E*). When *coE75A/C* was co-transfected with *E75A*, *E75B*, or *E75C*, only coE75A/C and E75B co-localized at the aggregating chromatin spots in HEK 293 cells (Fig. 6*F*). When *coE75A/B/C* was co-transfected with *E75A*, *E75B*, or *E75C*, the two proteins always co-localized at the aggregating chromatin spots (Fig. 6*G*). Nevertheless, E75noN had no co-localization with E75A/C but co-localized with E75B (Fig. 6*H*). The immunocytochemistry experiments demonstrated that the incomplete DBD in E75B is indispensable for the association between E75B and E75A/C. Furthermore, in HEK 293 cells, co-transfection with *coE75A/B/C* antagonized the transactivation ability of *E75A/C* in a dose-dependent manner (Fig. 6, *I* and *I′*), indicating that the incomplete DBD in E75B mediates physical interactions and thus the opposing actions between E75B and E75A/C.

Discussion

E75A/C Is a Bona Fide Transcription Factor That Induces Halloween Gene Expression—The majority of research on *Drosophila* focused on showing that E75 is a transcriptional repressor of HR3 through physical interaction and competing for ROREs. Nevertheless, HR3 inhibition is not able to explain the isoform-specific phenotypes of *E75* mutants. Here, we demonstrate for the first time that, in addition to HR3 inhibition, E75A/C is a *bona fide* transcription factor that directly drives Halloween gene expression and thus induces ecdysteroid biosynthesis. First, *E75* RNAi resulted in a decrease in expression of all five Halloween genes responsible for ecdysteroid biosynthesis, low ecdysteroid titers, impaired 20E signaling, repressed fat body remodeling, and lethality during metamorphosis (Figs. 1 and 2*A*). These E75 RNAi silkworms exhibit phenotypic defects similar to *Drosophila E75A* and *E75C* null mutants (9). Second, *E75A/C* overexpression up-regulates Halloween genes, promotes ecdysteroid biosynthesis, and accelerates metamorphosis (Fig. 3). Consistently, these phenotypes are similar to those observed after overexpression of *E75A* specifically in the *Drosophila* prothoracic glands (21). Third, dual-luciferase assays and ChIP-qPCR experiments together showed that E75A/C binds to ROREs in the Halloween gene promoter regions and thus induces expression of these genes (Fig. 4 and supplemental Fig. S3), providing strong evidence that E75A/C is a *bona fide* transcription activator. By binding to ROREs, E75A/C might act as a transcriptional repressor for competing with HR3, but more importantly, E75A/C functions as a transcriptional activator that induces Halloween gene expression. Fourth, E75B processes an incomplete DBD, which is not able to bind to ROREs to induce Halloween gene expression but still can inhibit HR3, confirming that Halloween gene induction and HR3 inhibition are indeed two separated functions of E75 (Figs. 3 and 4). Finally, in both the prothoracic glands and fat body of *Bombyx*, the *E75* mRNA levels peak nearly 1 day earlier than that of *HR3* (supplemental Fig. S5, *A, B, D,* and *E*) (25), implying that E75A/C functions as a transcriptional activator in inducing Halloween gene expression during the wandering stage and then as transcriptional repressor of HR3 during the larval-pupal transition. Regarding the ultimate regulation of ecdysteroid biosynthesis, E75 should first function directly and then act through inhibition of HR3 (supplemental Fig. S5*H*). Conclusively, E75A/C is a *bona fide* transcription activator that drives Halloween gene expression and thus induces ecdysteroid biosynthesis (Fig. 7).

E75B Antagonizes E75A/C to Regulate Halloween Gene Expression—The second important discovery of this study is on E75B antagonism of the transactivation ability of E75A/C to regulate Halloween gene expression. First, in contrast to *E75A/C* overexpression, *E75B* overexpression down-regulates Halloween genes, reduces ecdysteroid biosynthesis, and delays metamorphosis (Fig. 3). Second, co-transfection of *E75B* antagonizes the transactivation ability of *E75A/C* both in *vitro* (Fig. 5, *A–D′*) and *in vivo* (Fig. 5, *E* and *F*). Third, knock-out of *E75B* exhibits phenotypes (Fig. 5, *G* and *H*) similar to those seen after *E75A/C* overexpression (Fig. 3, *A* and *A′*). Finally and most importantly, the incomplete DBD of E75B mediates physical interactions and thus opposing actions between E75B and E75A/C (Fig. 6). Different from that, all E75 isoforms utilize their common C terminus to interact with and to antagonize transcriptional activity of HR3 (19, 26).

It is necessary to note that the *E75B* mutants in both *Drosophila* (9) and *Bombyx* (Fig. 5, *G* and *H*) are viable and fertile. Taking advantage of the comparatively long life cycle and the newly developed CRISPR/Cas9-mediated genome editing method in *Bombyx*, we have observed accelerated wandering behavior and elevated ecdysteroid titers in the *Bombyx E75B* mutants. Because E75B antagonizes transcriptional activity of both HR3 and E75A/C, the outcome of E75B in tuning ecdysteroid biosynthesis and developmental timing should be context-specific. We assume that this role of E75B should be

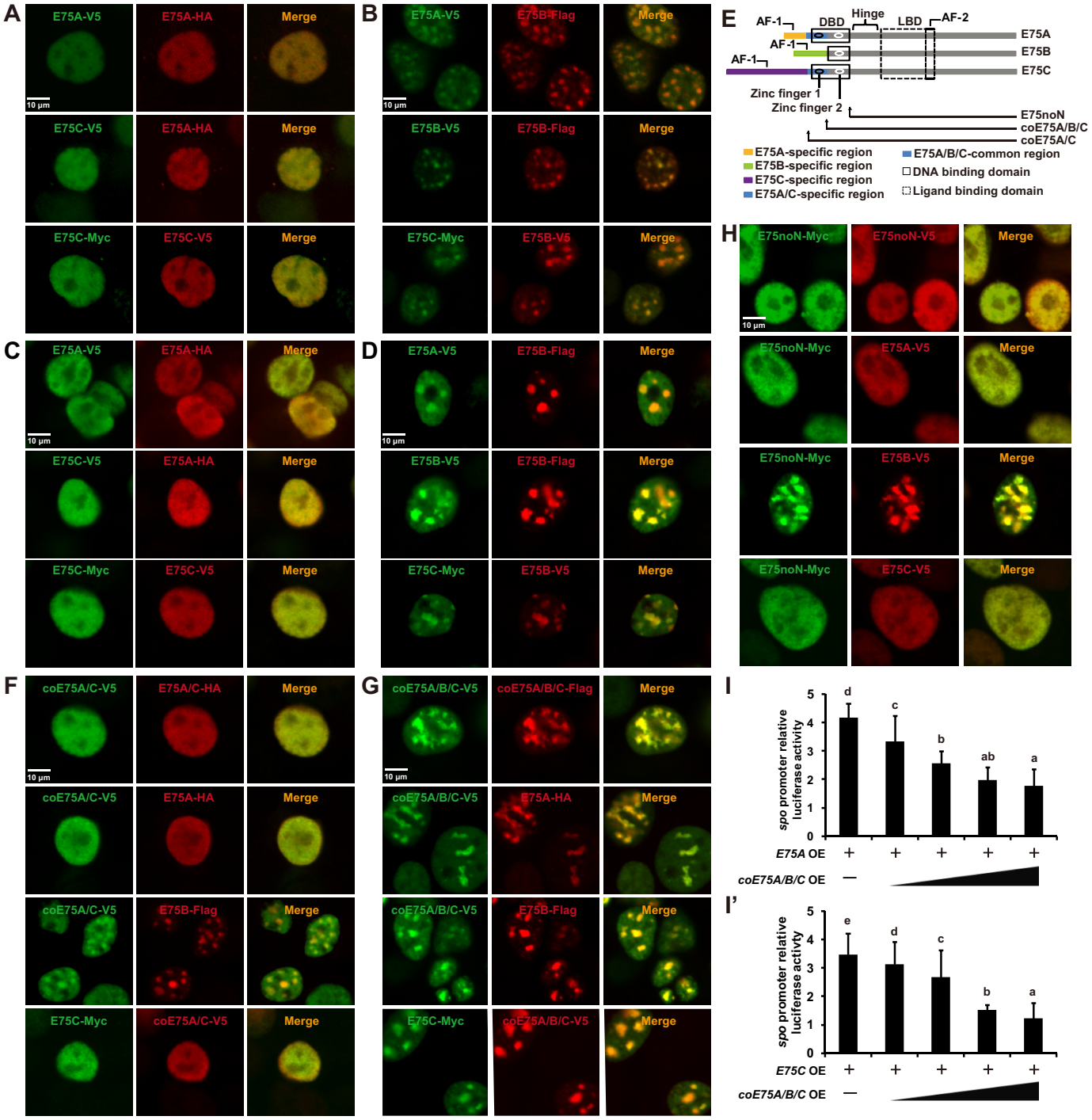

FIGURE 6. **Incomplete DBD in E75B mediates physical interactions and thus the opposing actions between E75B and E75A/C.** *A,* HEK 293 cells were co-transfected with two pcDNA 3.1(+) vectors expressing *E75A* and *E75C*, the C termini of which were fused to different tags (V5, HA, and Myc) for 48 h, and then immunocytochemistry was performed. *B,* co-transfection and immunocytochemistry in HEK 293 cells were performed as described in *A*, except that two pcDNA 3.1(+) vectors expressing *E75B* and *E75A* (or *E75C*), the C termini of which were fused to different tags (V5, FLAG, and Myc), were used. *C,* BmN cells were co-transfected with two pIEx-4 vectors expressing *E75A* and *E75C*, the C termini of which were fused to different tags (V5, HA, and Myc) for 48 h, and then immunocytochemistry was performed. *D,* co-transfection and immunocytochemistry in BmN cells were performed as described in *C*, except that two pIEx-4 vectors expressing *E75B* and *E75A* (or *E75C*), the C termini of which were fused to different tags (V5, FLAG, and Myc), were used. *E,* diagram showing three *E75* mutant constructs: *coE75A/C*, *coE75A/B/C*, and *E75noN*. *F–H,* co-transfection and immunocytochemistry in HEK 293 cells were performed described as in *A*, except that two pcDNA 3.1(+) vectors expressing *coE75A/C* (*F*) (or *coE75A/B/C* (*G*) or *E75noN* (*H*)) and *E75A/B/C*, the C termini of which were fused to different tags (V5, HA, FLAG, and Myc), were used. *I* and *I′*, HEK 293 cells were co-transfected with three expression constructs (*coE75A/B/C*, *E75A/E75C*, and *EGFP*), the pGL3 basic plasmids containing ~2.5-kb promoter regions of *spo* and the *hsp70* basal promoter regulating expression of firefly luciferase (*Fluc*), and a reference reporter plasmid carrying *Renilla* luciferase (*Rluc*) for 48 h of transfection, and then the dual-luciferase assays were performed. Luciferase activity fold change is defined as the relative luciferase activity compared with *EGFP*.

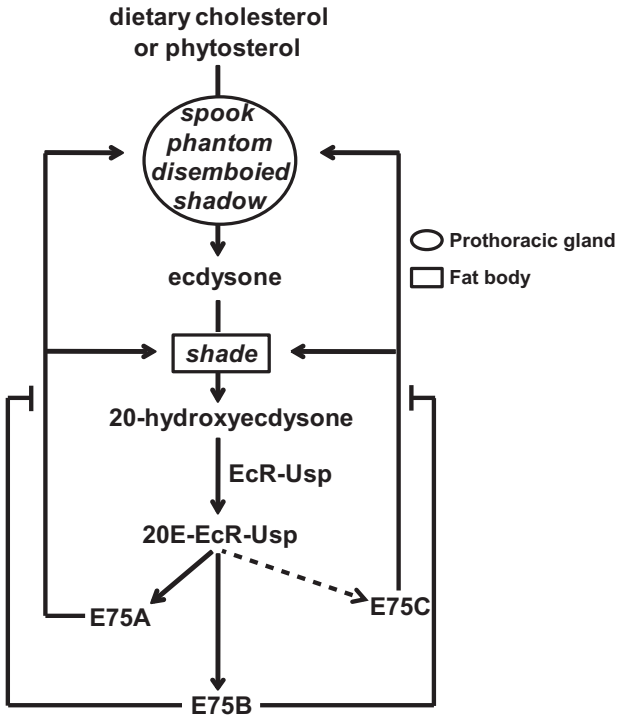

FIGURE 7. **A model, E75 isoforms mediate a fine regulatory loop between ecdysteroid biosynthesis and 20E signaling.** 20E rapidly induces the expression of *E75A* and *E75B*, whereas its induction of *E75C* expression is slow. E75A/C induces the Halloween gene expression responsible for ecdysone biosynthesis in the prothoracic glands and the conversion from ecdysone to 20E in the fat body, whereas E75B antagonizes the transactivation ability of E75A/C. This model supports the central role of the 20E-response gene *E75* in regulating ecdysteroid biosynthesis. The E75-mediated regulatory loop represents a fine autoregulation of steroidogenesis which contributes to the precise control of developmental timing.

conserved in *Bombyx* and *Drosophila*, because the protein structure and 20E induction of expression are the same in both animals. Previous studies also showed that *E75A* and *E75B* have opposing effects on the apoptosis/development choice of the egg chamber in *Drosophila* (22). Similarly, *E75* isoforms play distinct roles in regulating female reproduction in the mosquito *A. aegypti* (23). These studies indicate that E75 isoforms have an isoform-specific function in regulating insect reproduction, in line with our findings that E75A/C and E75B oppositely regulate Halloween gene expression, ecdysteroid biosynthesis, and developmental timing. In conclusion, lacking a complete DBD, E75B does not act as an independent transcription activator, but antagonizes the transactivation ability of E75A/C by binding to and changing the conformation of E75A/C (Fig. 7).

Correlations among E75, HR3, and NO—Phylogenetic analysis reveals that E75 and HR3 belong to NR subfamily 1 and are closely related (3). Multiple lines of evidence support that, by physical interaction and by competing for ROREs, E75 isoforms indiscriminately act as transcriptional repressors for HR3. By being either transcriptional repressors for HR3 in relieving HR3 inhibition on Halloween gene expression (21) or transcriptional activators in inducing Halloween gene expression (Figs. 1–4), E75A/C ultimately promotes ecdysteroid biosynthesis and developmental transition (supplemental Fig. S5*H*). Both gain-of-function and loss-of-function results clearly show that E75B inhibits Halloween gene expression and thus ecdysteroid biosynthesis *in vivo* (Figs. 3 and 5), indicating that its inhibition of ecdysteroid biosynthesis via antagonizing the transactivation ability of E75A/C (Figs. 3–6) is more crucial than its possible promotion via relieving HR3 inhibition (supplemental Fig. S5 *H*).

NO and CO are able to reverse the ability of E75 to interfere with HR3; thus, the function of E75 in counteracting HR3 might vary depending on the availability of these gases. We investigated the developmental profiles of NO synthetase (*NOS1* and *NOS2*) in the prothoracic glands and the fat body in *Bombyx*. Interestingly, the expression peaks of *NOS1* and *NOS2* (supplemental Fig. S5, *C, C′, F,* and *F′*) never match that of *E75* (supplemental Fig. S5, *A, B, D,* and *E*). Moreover, the transcriptional activity of E75A/C in inducing Halloween gene expression was able to be reversed by NO (supplemental Fig. S5*G*). Thus, the ability of E75A/C to promote ecdysteroid biosynthesis and developmental transition could be reversed by NO (supplemental Fig. S5 *H*). We suppose that the binding of E75B with E75A/C, the binding of all E75 isoforms with HR3, and the binding of NO with all E75 isoforms will result in changes of conformation and transactivation ability of the latter ones.

E75-mediated Steroidogenesis Autoregulation Contributes to the Precise Control of Developmental Timing—Steroidogenesis autoregulation in insects involves a fine regulatory loop between ecdysteroid biosynthesis and 20E signaling. A number of genes in the 20E-triggered transcriptional cascade regulate ecdysone biosynthesis and thus ecdysteroid titers in both *Drosophila* and *Bombyx* (9, 21, 31–33). βftz-F1, Br-C, HR3, and E75 regulate Halloween gene expression in the prothoracic glands (21, 32, 33). Here, we found that E75 binds to ROREs and induces Halloween gene expression (Fig. 4 and supplemental Fig. S3). Importantly, E75A/C induces the Halloween gene expression that is responsible for not only ecdysone biosynthesis in the prothoracic glands but also the conversion from ecdysone to 20E in the fat body, whereas E75B has opposing roles. The composite data support the central role of E75 in the regulatory loop of ecdysteroid biosynthesis (Fig. 7).

In summary, 20E induces the expression of *E75* isoforms differently, and E75A/C and E75B oppositely regulate ecdysteroid biosynthesis, forming a fine regulatory loop between ecdysteroid biosynthesis and 20E signaling (Fig. 7). Acting independently or through HR3 inhibition in a context-specific manner, E75 isoforms are involved in the fine regulation of ecdysteroid biosynthesis, which contributes to the precise control of developmental timing (supplemental Fig. S5*H*). This study provides a paradigm for how NR isoforms accurately mediate steroidogenesis autoregulation and thus developmental timing in animals.

Experimental Procedures

Silkworms and Cells—Bombyx larvae (p50 strain) were provided by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China), and fed fresh mulberry leaves at 25 °C under 14-h light/10-h dark cycles (25, 29). Bm-N cells were maintained in TC-100 medium (PAN-BIOTECH, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Gibco). HEK 293 cells were maintained in

Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% fetal bovine serum (25, 31).

E75 RNAi in Bombyx Larvae—The *E75* dsRNA (25) was synthesized using a T7 RiboMAX™ Express RNAi kit (Promega, P1700). The *EGFP* dsRNA was used as a control. Thirty μg of dsRNA per larva was injected at IW. The prothoracic glands, peripheral fat body tissues from the 5th abdominal segment, and hemolymph samples were collected at the indicated times for further analysis (25).

Baculovirus-mediated Overexpression of E75 Isoforms in Bombyx Larvae—Using the homologous recombination technique (39), we generated the BmNPV *egt* mutant that allows silkworms, which survive until pupation, to produce sufficient E75 protein. The BmNPVs expressing *E75A, E75B*, and *E75C* were obtained in the same manner as the *Autographa californica* nucleopolyhedrovirus (25). Five μl of P2 BmNPV (~10^5 pfu) was injected into each *Bombyx* larva on L5D2, and then the prothoracic glands, fat body, and hemolymph were collected at the indicated times for further analysis.

CRISPR/Cas9-mediated Knock-out of E75B in Bombyx—Our colleagues previously developed efficient approaches for CRISPR/Cas9-mediated genome editing in *Bombyx* (37, 38) and helped us to perform the *E75B* knock-out experiment in this study. Cas9 mRNA (mMESSAGE mMACHINE kit, Ambion, Austin, TX) and *E75B* sgRNA (MAXIscript T7 Kit, Ambion, Austin, TX), TAATACGACTCACTATAGGTGCTAGTGAGCATGCTGGAGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT, was synthesized and purified separately. A mixture of Cas9 mRNA (300 ng/μl) and *E75B* sgRNA (300 ng/μl; with *EGFP* sgRNA as a control) was injected into the non-diapause preblastoderm p50 embryos prepared within 6 h after oviposition using a micro-injector (Narishige, Tokyo, Japan), and then the embryos were incubated at 25 °C in a humidified chamber for 10–12 days until larval hatching. Approximately 24 h after IW, genomic DNA was extracted for mutagenesis analysis. The prothoracic glands, fat body, and hemolymph were collected for further analysis.

Conventional Molecular, Biochemical, and Cellular Methods—Details of caspase-3 activity measurement, qPCR, and Western blotting have been previously described (25, 31, 40). Production of the EcR, Met1, Br-C, and E75 antibodies have been reported in our publications (25, 30). The AB11 USP antibody was a kind gift from Dr. Fotis Kafatos. The Western blotting images were obtained with a Tanon-5500 Chemiluminescent Imaging System (Tanon, China).

Fluorescence Microscopy and Transmission Electron Microscopy—The prothoracic glands and fat body were dissected and processed for fluorescence microscopy and transmission electron microscopy analyses as described previously (25, 29, 31, 40). TUNEL (Beyotime, China) labeling and LysoTracker Red (Invitrogen) staining were used to estimate caspase activity and autophagy, respectively. Cell death was also detected by propidium iodide staining (red nuclei) and nuclei with Hoechst 33342 (blue) (Beyotime). Pictures were taken under an FV10-ASW confocal microscope (Olympus, Japan) at ×40 magnification, and each type of observation was performed under the same conditions. A H7650 transmission electron microscope (Hitachi, Japan) was used to observe autophagic components, mitochondria, and other cell structures.

Tissue Culture and rpHPLC-EIA Measurements of Ecdysteroids—For measurements of ecdysteroid titers in the hemolymph, we used EIA (Cayman Chemical) (38). In some cases, ecdysone and 20E in the hemolymph were separated using a modified rpHPLC procedure (41) followed by quantification using EIA. In brief, total ecdysteroids in the hemolymph samples were extracted with methanol, dried, and re-dissolved in 20% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). An Agilent 1100 Series HPLC system (Agilent Technologies) equipped with a variable UV wavelength detector (set at 240 nm) was employed. All samples were separated by an Eclipse Plus C18 (4.6 × 250 mm) column (Agilent Technologies) using a variable mobile phase consisting of 20% ACN containing 0.1% TFA for 5 min and a linear gradient of 20–80% ACN containing 0.1% TFA for 20 min. The flow rate was 1 ml/min. 20E and ecdysone standards (Sigma) were eluted after 7 and 14 min, respectively. All sample fractions were collected at 6.7–8.7 and 13–15 min for 20E and ecdysone, respectively, dried, re-dissolved in EIA buffer, and measured by EIA. The ratio of ecdysone and 20E was calculated.

For the measurement of ecdysone release, the prothoracic glands were dissected out and cultured in Grace's medium (Sigma) at 25 °C. After pre-incubation for 1 h, the medium was replaced with fresh medium. Four hours after incubation, the medium was collected, dried, and re-dissolved in EIA buffer, and the ecdysteroid concentration was determined by EIA. To measure the conversion from ecdysone to 20E, the fat body was cultured in Grace's medium at 25 °C. After pre-incubation for 1 h, the medium was replaced with fresh medium containing 5 μM ecdysone. Four hours after incubation, the medium was collected and concentrated. Ecdysone and 20E in the medium were separated by rpHPLC, and the fractions were dried, re-dissolved in EIA buffer, and measured by EIA. The conversion from ecdysone to 20E was calculated.

ChIP Assay in Bm-N Cells—The modified pIEx-4 vector containing the BmNPV *ie1* promoter (42) was used to overexpress *E75A/B/C-V5* in Bm-N cells. Bm-N cells were grown in 10-cm dishes (70% confluent) and transfected with the *E75A/B/C-V5* expression plasmid for 48 h using the Effectene transfection reagent (Qiagen, Germany). Then, the cells were fixed and subjected to ChIP assay (31, 42, 43) using the agarose ChIP kit (Pierce) and the V5 antibody (Sigma). Mock immunoprecipitations with pre-immune serum were used for negative controls. The precipitated DNA and input were analyzed by qPCR to detect the binding between E75A/B/C-V5 and ROREs in promoter regions and CDS regions (as negative control) of the five Halloween genes.

Dual-Luciferase Assay in HEK 293 Cells and BmN Cells—To examine whether the promoter regions of the five Halloween genes are responsive to E75, the 2.5-kb regions of each Halloween gene promoter upstream of the transcription start site (or the RORE-deleted mutant constructs) were cloned into the pGL3 basic vector containing the *hsp70* minimal promoter (Promega). The pRL vector (Promega) carrying *Renilla* luciferase driven by the *Actin3* promoter was used for normalization. *E75A/B/C* (or *coE75A/B/C*) was cloned into the pcDNA 3.1(+)

vector (Invitrogen) to create the expression constructs. After co-transfection of *E75A/B/C* expression construct, a reporter pGL3 vector, and the reference pRL vector into HEK 293 cells for 48 h using the Effectene transfection reagent (Qiagen), the cells were collected. The relative luciferase activity was calculated by normalizing the reporter firefly luciferase level to the reference *Renilla* luciferase level. Dual-luciferase assays were conducted using the dual-luciferase assay system (Promega) and a Modulus luminometer (Turner BioSystems) (29, 31, 42, 43). For some experiments, two constructs of *EGFP*, *E75A*, *E75B*, *E75C*, or *coE75A/B/C* were co-transfected into HEK 293 cells equally or in a dose-dependent manner. When necessary, the NO donor, 2,2′-(hydroxynitrosohydrazino)bis-ethanimine (Sigma; 200 μM) was added to the medium (10). Dual-luciferase assays in BmN cells were performed the same as in HEK 293 cells except the expression vector was pIEx-4 containing the BmNPV *ie1* promoter as above described.

Cytohistochemistry in HEK 293 Cells and BmN Cells—Microscope coverslips (Fisher, 12-542A) were sterilized before use and placed into 6-well plates during HEK 293 cell plating. After 1 day of pre-incubation, the cells were co-transfected with two pcDNA 3.1(+) vectors expressing *E75A*, *E75B* (or its mutants *coE75A/C*, *coE75A/B/C*, and *E75noN*), or *E75C*, the C termini of which were fused to different tags (V5, HA, FLAG, and Myc), for 48 h. After extensive washing, the coverslips were fixed in 4% paraformaldehyde for 45 min at room temperature, blocked in phosphate-buffered saline containing 5% BSA and 1% Triton X-100 (PBSBT) for 1 h, and incubated with two different primary tag antibodies (V5, HA, FLAG, and Myc, Sigma) (diluted 1:200) at 4 °C overnight. The coverslips were washed for 1 h in PBSBT and incubated with two counterpart FITC green/red-conjugated secondary antibodies from mouse/rabbit (diluted 1:200) for 2 h at room temperature (43). Images were captured using the Olympus FV10-ASW confocal microscope at ×40 magnification. Cytohistochemistry in BmN cells were performed the same as in HEK 293 cells except the expression vector was pIEx-4 containing the BmNPV *ie1* promoter as described above.

Statistics—The experimental data were analyzed using Student's *t* test and analysis of variance. For the *t* test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. For analysis of variance, bars labeled with different lowercase letters are significantly different ($p < 0.05$). Throughout the study, values are represented as the mean ± S.D. of five independent experiments.

Author Contributions—S. L. conceived and designed the experiments. K. L., L. T., and S. Y. G. performed the research. Y. C., Z. J. G., J. Z. Z., and S. H. G. contributed important resources, techniques, and reagents. K. L. and S.L. analyzed the data. S. L., K. L., and S. R. P. wrote the paper.

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References

1. Riddiford, L. M., Cherbas, P., and Truman, J. W. (2000) Ecdysone receptors and their biological actions. *Vitam. Horm.* **60,** 1–73
2. Yin, V. P., and Thummel, C. S. (2005) Mechanisms of steroid-triggered programmed cell death in *Drosophila*. *Semin. Cell Dev. Biol.* **16,** 237–243
3. King-Jones, K., and Thummel, C. S. (2005) Nuclear receptors–a perspective from *Drosophila*. *Nat. Rev. Genet.* **6,** 311–323
4. Evans, R. M., and Mangelsdorf, D. J. (2014) Nuclear receptors, RXR, and the big bang. *Cell* **157,** 255–266
5. Cheng, D., Xia, Q., Duan, J., Wei, L., Huang, C., Li, Z., Wang, G., and Xiang, Z. (2008) Nuclear receptors in *Bombyx mori*: insights into genomic structure and developmental expression. *Insect Biochem. Mol. Biol.* **38,** 1130–1137
6. Kostrouchova, M., and Kostrouch, Z. (2015) Nuclear receptors in nematode development: natural experiments made by a phylum. *Biochim. Biophys. Acta* **1849,** 224–237
7. Feigl, G., Gram, M., and Pongs, O. (1989) A member of the steroid hormone receptor gene family is expressed in the 20-OH-ecdysone inducible puff 75B in *Drosophila melanogaster*. *Nucleic Acids Res.* **17,** 7167–7178
8. Segraves, W. A., and Hogness, D. S. (1990) The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Dev.* **4,** 204–219
9. Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W. A., and Thummel, C. S. (2002) Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Dev. Cell* **3,** 209–220
10. Reinking, J., Lam, M. M., Pardee, K., Sampson, H. M., Liu, S., Yang, P., Williams, S., White, W., Lajoie, G., Edwards, A., and Krause, H. M. (2005) The *Drosophila* nuclear receptor e75 contains heme and is gas responsive. *Cell* **122,** 195–207
11. Cáceres, L., Necakov, A. S., Schwartz, C., Kimber, S., Roberts, I. J., and Krause, H. M. (2011) Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. *Genes Dev.* **25,** 1476–1485
12. Aicart-Ramos, C., Valhondo Falcón, M., Ortiz de Montellano, P. R., and Rodriguez-Crespo, I. (2012) Covalent attachment of heme to the protein moiety in an insect E75 nitric oxide sensor. *Biochemistry* **51,** 7403–7416
13. Harding, H. P., and Lazar, M. A. (1995) The monomer-binding orphan receptor Rev-Erb represses transcription as a dimer on a novel direct repeat. *Mol. Cell. Biol.* **15,** 4791–4802
14. Raghuram, S., Stayrook, K. R., Huang, P., Rogers, P. M., Nosie, A. K., McClure, D. B., Burris, L. L., Khorasanizadeh, S., Burris, T. P., and Rastinejad, F. (2007) Identification of heme as the ligand for the orphan nuclear receptors REV-ERBα and REV-ERBβ. *Nat. Struct. Mol. Biol.* **14,** 1207–1213
15. Pardee, K. I., Xu, X., Reinking, J., Schuetz, A., Dong, A., Liu, S., Zhang, R., Tiefenbach, J., Lajoie, G., Plotnikov, A. N., Botchkarev, A., Krause, H. M., and Edwards, A. (2009) The structural basis of gas-responsive transcription by the human nuclear hormone receptor REV-ERBβ. *PLoS Biol.* **7,** e43
16. Bernardo, T. J., Dubrovskaya, V. A., Jannat, H., Maughan, B., and Dubrovsky, E. B. (2009) Hormonal regulation of the E75 gene in *Drosophila*: identifying functional regulatory elements through computational and biological analysis. *J. Mol. Biol.* **387,** 794–808
17. Buszczak, M., Freeman, M. R., Carlson, J. R., Bender, M., Cooley, L., and Segraves, W. A. (1999) Ecdysone response genes govern egg chamber development during mid-oogenesis in *Drosophila*. *Development* **126,** 4581–4589
18. White, K. P., Hurban, P., Watanabe, T., and Hogness, D. S. (1997) Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science* **276,** 114–117
19. Lam, G. T., Jiang, C., and Thummel, C. S. (1997) Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. *Development* **124,** 1757–1769
20. Lam, G., Hall, B. L., Bender, M., and Thummel, C. S. (1999) DHR3 is required for the prepupal-pupal transition and differentiation of adult structures during *Drosophila* metamorphosis. *Dev. Biol.* **212,** 204–216
21. Parvy, J. P., Wang, P., Garrido, D., Maria, A., Blais, C., Poidevin, M., and Montagne, J. (2014) Forward and feedback regulation of cyclic

steroid production in *Drosophila melanogaster*. *Development* **141,** 3955–3965
22. Terashima, J., and Bownes, M. (2006) E75A and E75B have opposite effects on the apoptosis/development choice of the *Drosophila* egg chamber. *Cell Death Differ.* **13,** 454–464
23. Cruz, J., Mane-Padros, D., Zou, Z., and Raikhel, A. S. (2012) Distinct roles of isoforms of the heme-liganded nuclear receptor E75, an insect ortholog of the vertebrate Rev-erb, in mosquito reproduction. *Mol. Cell. Endocrinol.* **349,** 262–271
24. Swevers, L., Eystathioy, T., and Iatrou, K. (2002) The orphan nuclear receptors BmE75A and BmE75C of the silkmoth *Bombyx mori*: hormonal control and ovarian expression. *Insect Biochem. Mol. Biol.* **32,** 1643–1652
25. Li, K., Guo, E., Hossain, M. S., Li, Q., Cao, Y., Tian, L., Deng, X., and Li, S. (2015) Bombyx E75 isoforms display stage- and tissue-specific responses to 20-hydroxyecdysone. *Sci. Rep.* **5,** 12114
26. Swevers, L., Ito, K., and Iatrou, K. (2002) The BmE75 nuclear receptors function as dominant repressors of the nuclear receptor BmHR3A. *J. Biol. Chem.* **277,** 41637–41644
27. Xia, Q., Li, S., and Feng, Q. (2014) Advances in silkworm studies accelerated by the genome sequencing of *Bombyx mori*. *Annu. Rev. Entomol.* **59,** 513–536
28. Tian, L., Guo, E., Diao, Y., Zhou, S., Peng, Q., Cao, Y., Ling, E., and Li, S. (2010) Genome-wide regulation of innate immunity by juvenile hormone and 20-hydroxyecdysone in the *Bombyx* fat body. *BMC Genomics* **11,** 549
29. Tian, L., Ma, L., Guo, E., Deng, X., Ma, S., Xia, Q., Cao, Y., and Li, S. (2013) 20-Hydroxyecdysone upregulates Atg genes to induce autophagy in the *Bombyx* fat body. *Autophagy* **9,** 1172–1187
30. Guo, E., He, Q., Liu, S., Tian, L., Sheng, Z., Peng, Q., Guan, J., Shi, M., Li, K., and Gilbert, L. I., Wang, J., Cao, Y., and Li, S. (2012) MET is required for the maximal action of 20-hydroxyecdysone during *Bombyx* metamorphosis. *PLoS ONE* **7,** e53256
31. Liu, X., Dai, F., Guo, E., Li, K., Ma, L., Tian, L., Cao, Y., Zhang, G., Palli, S. R., and Li, S. (2015) 20-hydroxyecdysone (20E) primary response gene E93 modulates 20E signaling to promote *Bombyx* larval-pupal metamorphosis. *J. Biol. Chem.* **290,** 27370–27383
32. Parvy, J. P., Blais, C., Bernard, F., Warren, J. T., Petryk, A., Gilbert, L. I., O'Connor, M. B., and Dauphin-Villemant, C. (2005) A role for βFTZ-F1 in regulating ecdysteroid titers during post-embryonic development in *Drosophila melanogaster*. *Dev. Biol.* **282,** 84–94
33. Moeller, M. E., Danielsen, E. T., Herder, R., O'Connor, M. B., and Rewitz, K. F. (2013) Dynamic feedback circuits function as a switch for shaping a maturation-inducing steroid pulse in *Drosophila*. *Development* **140,** 4730–4739
34. Gilbert, L. I., Rybczynski, R., and Warren, J. T. (2002) Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol.* **47,** 883–916
35. Rewitz, K. F., O'Connor, M. B., and Gilbert, L. I. (2007) Molecular evolution of the insect Halloween family of cytochrome P450s: phylogeny, gene organization and functional conservation. *Insect Biochem. Mol. Biol.* **37,** 741–753
36. Niwa, R., and Niwa, Y. S. (2014) Enzymes for ecdysteroid biosynthesis: their biological functions in insects and beyond. *Biosci. Biotechnol. Biochem.* **78,** 1283–1292
37. Wang, Y., Li, Z., Xu, J., Zeng, B., Ling, L., You, L., Chen, Y., Huang, Y., and Tan, A. (2013) The CRISPR/Cas system mediates efficient genome engineering in *Bombyx mori*. *Cell Res.* **23,** 1414–1416
38. Li, Z., You, L., Zeng, B., Ling, L., Xu, J., Chen, X., Zhang, Z., Palli, S. R., Huang, Y., and Tan, A. (2015) Ectopic expression of ecdysone oxidase impairs tissue degeneration in *Bombyx mori*. *Proc. Biol. Sci.* **282,** 20150513
39. Guo, Z. J., Qiu, L. H., An, S. H., Yao, Q., Park, E. Y., Chen, K. P., and Zhang, C. X. (2010) Open reading frame 60 of the *Bombyx mori* nucleopolyhedrovirus plays a role in budded virus production. *Virus Res.* **151,** 185–191
40. Liu, Y., Sheng, Z., Liu, H., Wen, D., He, Q., Wang, S., Shao, W., Jiang, R. J., An, S., Sun, Y., Bendena, W. G., Wang, J., Gilbert, L. I., Wilson, T. G., Song, Q., and Li, S. (2009) Juvenile hormone counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent programmed cell death in *Drosophila*. *Development* **136,** 2015–2025
41. Marchal, E., Verlinden, H., Badisco, L., Van Wielendaele, P., and Vanden Broeck, J. (2012) RNAi-mediated knockdown of Shade negatively affects ecdysone-20-hydroxylation in the desert locust, *Schistocerca gregaria*. *J. Insect Physiol.* **58,** 890–896
42. Hossain, M. S., Liu, Y., Zhou, S., Li, K., Tian, L., and Li, S. (2013) 20-Hydroxyecdysone-induced transcriptional activity of FoxO upregulates brummer and acid lipase-1 and promotes lipolysis in *Bombyx* fat body. *Insect Biochem. Mol. Biol.* **43,** 829–838
43. He, Q., Wen, D., Jia, Q., Cui, C., Wang, J., Palli, S. R., and Li, S. (2014) Heat shock protein 83 (Hsp83) facilitates methoprene-tolerant (Met) nuclear import to modulate juvenile hormone signaling. *J. Biol. Chem.* **289,** 27874–27885

Supplementary Figures and Figure Legends:

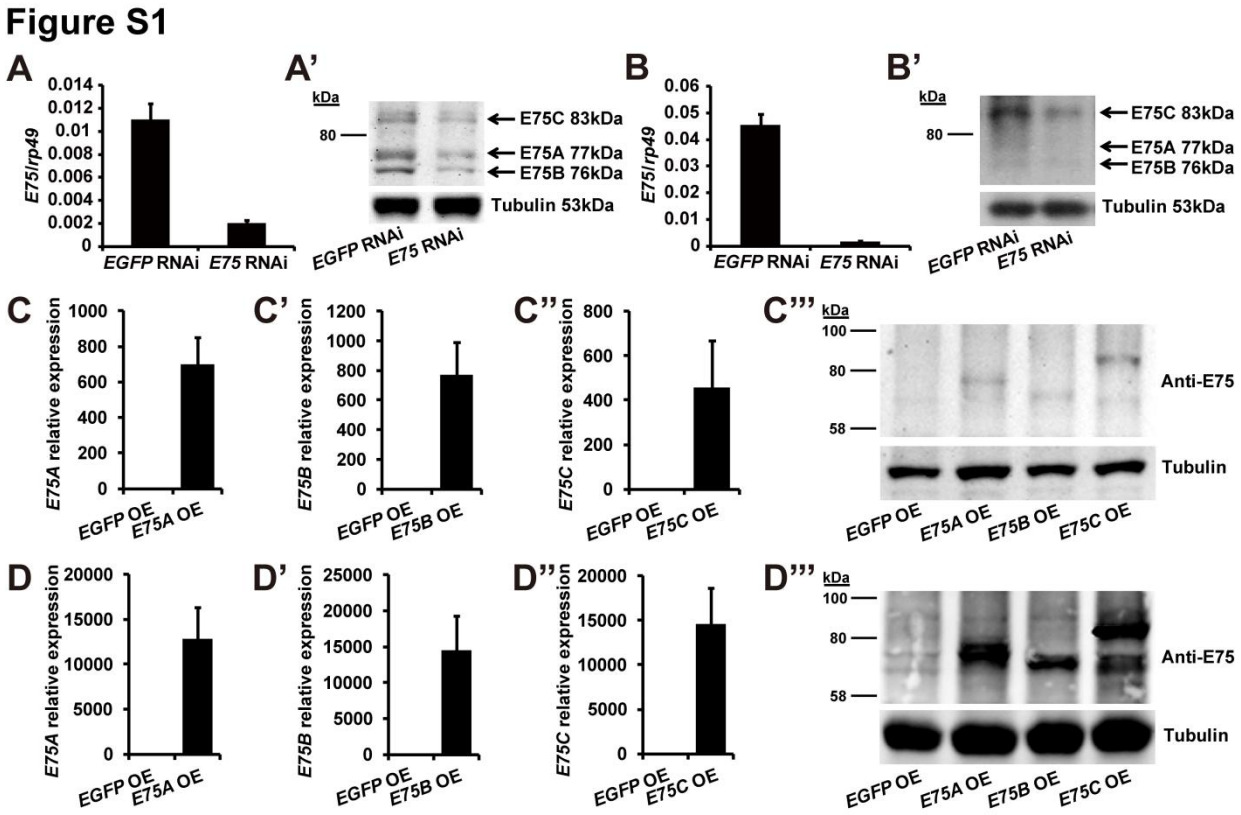

Figure S1. E75 mRNA and protein levels after *E75* RNAi and overexpression

E75 dsRNA (30 μg per larva) was injected into each *Bombyx* larva at the initiation of the wandering stage. *EGFP* dsRNA was used as a control.

(A and A') E75 mRNA (A) and protein (A') levels in the fat body.

(B and B') E75 mRNA (B) and protein (B') levels in the prothoracic glands.

P2 BmNPV *egt* mutant expressing *E75A/B/C* (5 μl; ~10^5 pfu) was injected into each *Bombyx* larva on day 2 of the fifth instar. BmNPV expressing *EGFP* was used as a control. The samples were analyzed 72 h after injection of BmNPV expressing *E75A/B/C*.

(C-C''') E75A/B/C mRNA (C-C'') and protein (C''') levels in the prothoracic glands.

(D-D''') E75A/B/C mRNA (D-D'') and protein (D''') levels in the fat body.

Figure S2

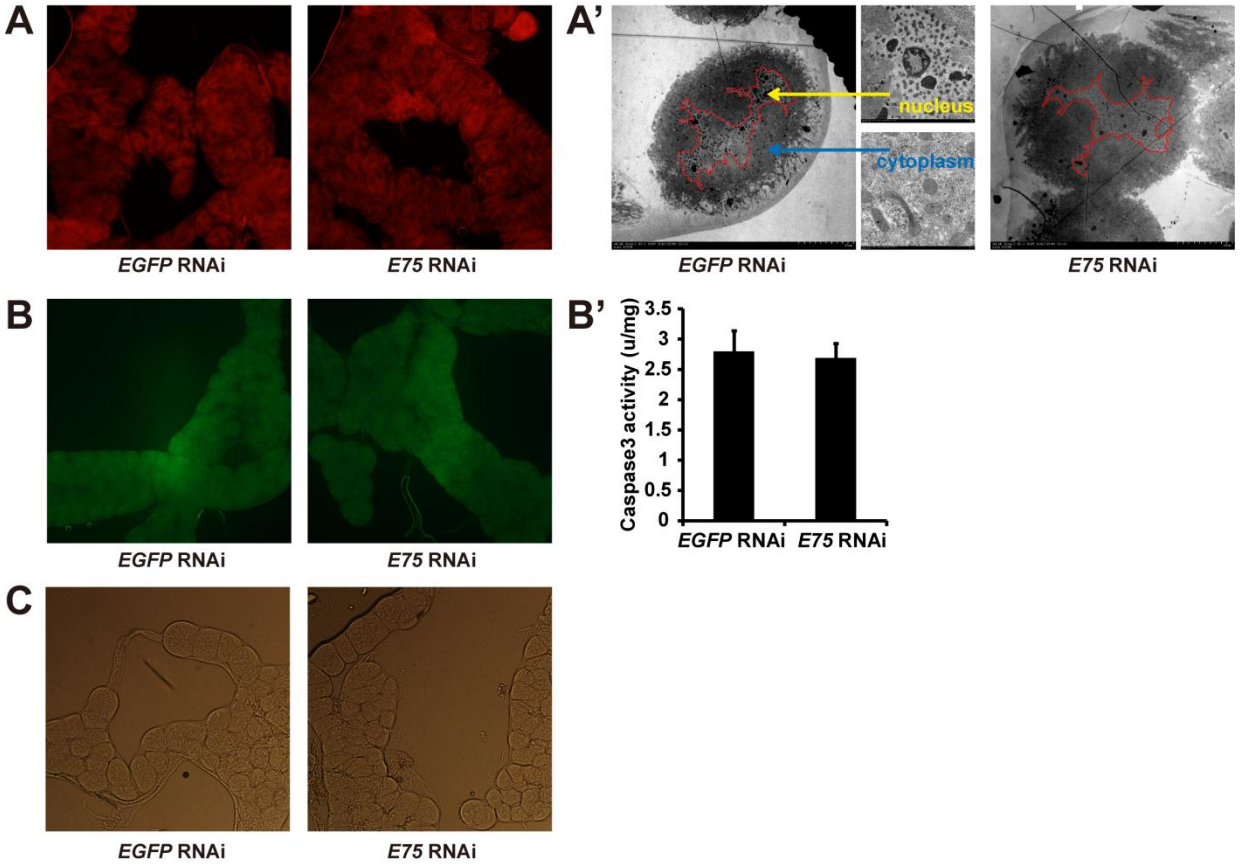

Figure S2. *E75* RNAi does not cause apparent morphologic changes in the prothoracic glands

E75 dsRNA (30 μg per larva) was injected into each *Bombyx* larva at the initiation of the wandering stage. *EGFP* dsRNA was used as a control.

(A and A') LysoTracker Red staining (red, 40×) (B) and TEM analysis (7,500×) (B') in the prothoracic glands 24 h after dsRNA treatment.

(B and B') TUNEL labeling (green, 40×) (B) and caspase 3 activity (B') in the prothoracic glands 24 h after dsRNA treatment.

(C) A comparison of the overall structure of the prothoracic glands under a light microscopy 24 h after dsRNA treatment.

Figure S3

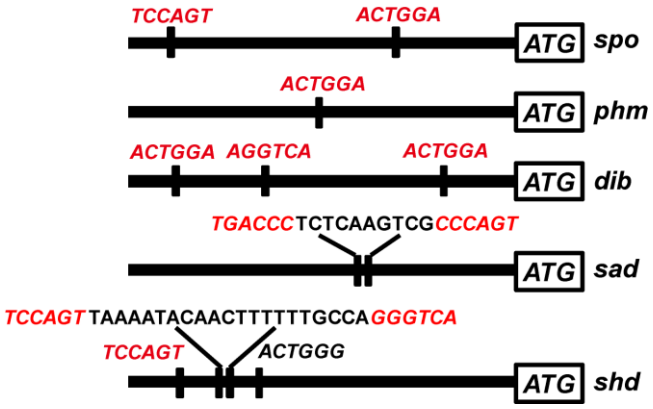

Figure S3. A diagram showing 2, 1, 3, 2, and 4 potential ROREs in the ~2.5-kb promoter regions of *spo*, *phm*, *dib*, *sad*, and *shd*, respectively

Figure S4

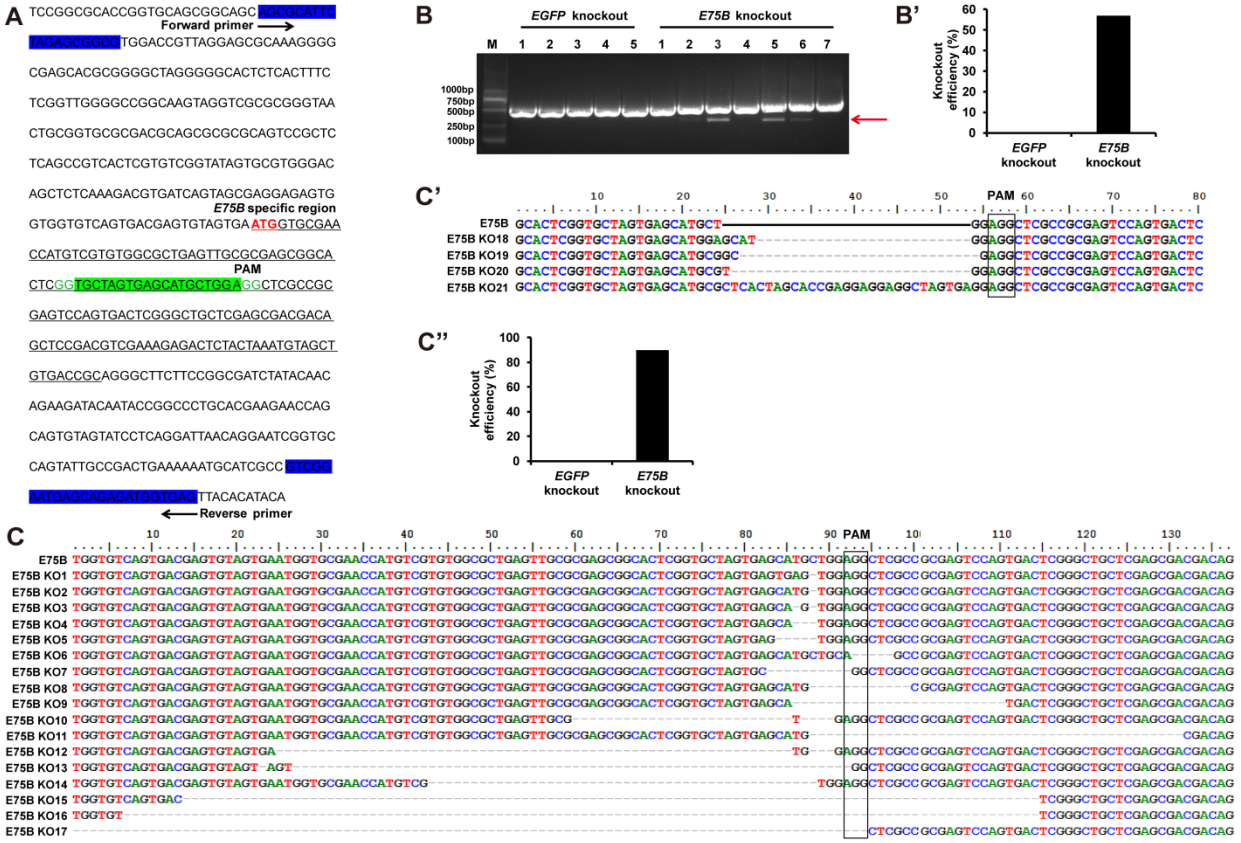

Figure S4. CRISPR/Cas9-mediated knockout of *E75B*

A mixture of Cas9 mRNA (300 ng/μl) and *E75B* sgRNA (300 ng/μl; with *EGFP* sgRNA as a control) was injected into the non-diapaused preblastoderm p50 embryos prepared within 6 h after oviposition. Approximately at 24 h after the initiation of the wandering stage, genomic DNA was extracted for mutagenesis analysis.

(A) The sequences between the two arrows indicate the amplicon nearly 550 bp from genomic DNA PCR from the control larvae. Codons in blue show the sequence for primer design, the 3 bp codons in red show the initiation codon of *E75B*, while the codons in green indicate the PAM region used for designing *E75B* sgRNA.

(B and B') An additional maximum deletion band nearly 260 bp (showed by the red arrow), which is apparently smaller than the control 550 bp band, was got by genomic DNA PCR from the *E75B*-knockout larvae (B). The chart (B') shows the quantification of the additional maximum deletion in (B).

(C-C'') The variation of *E75B* knockout including deletion (C) (note E75B KO17 is the additional maximum deletion in B) and insertion (C'). The chart (C'') shows the quantification of successful *E75B* knockout larvae.

Figure S5

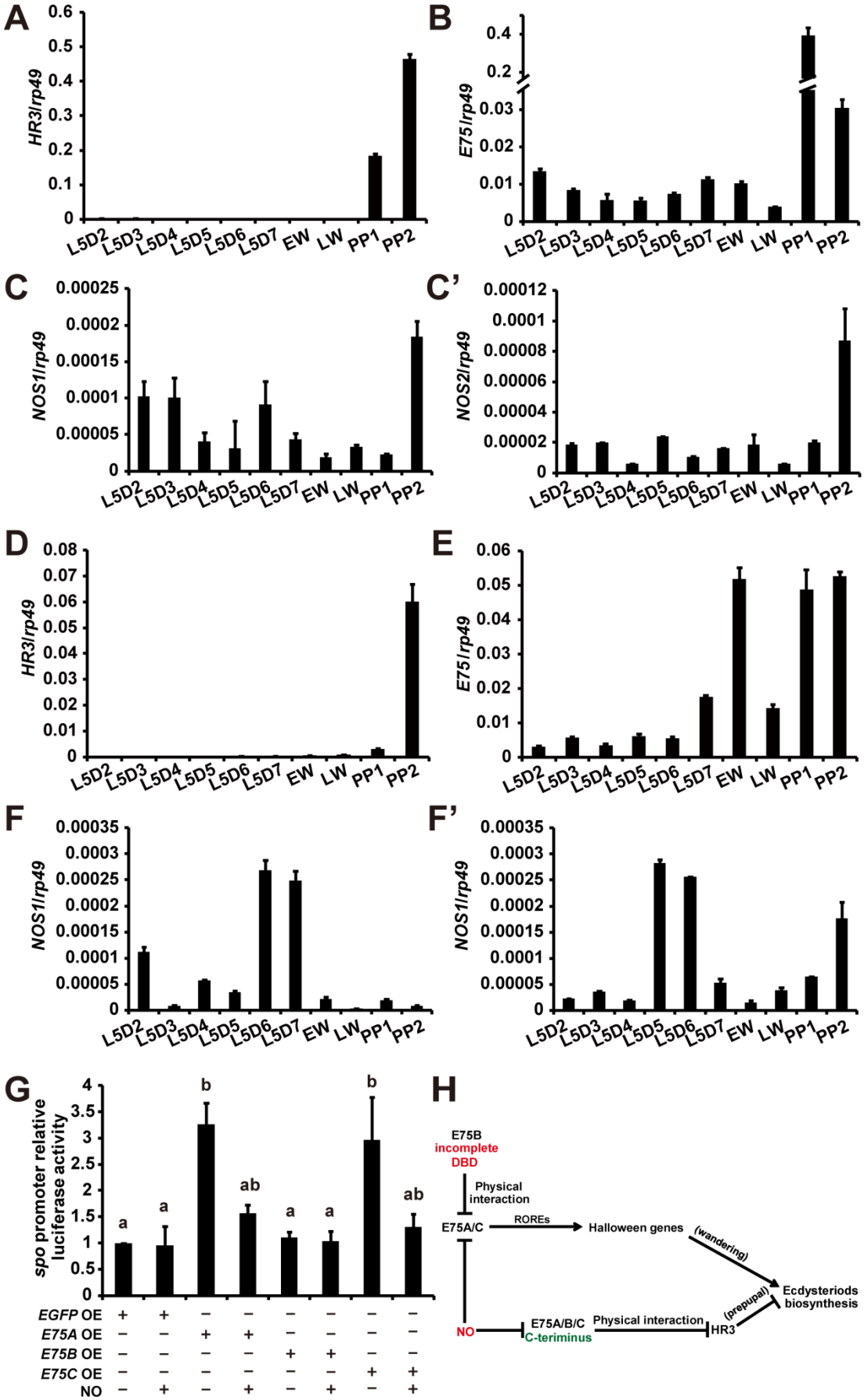
A
HR3/rp49
B
E75/rp49
C
NOS1/rp49
C'
NOS2/rp49
D
HR3/rp49
E
E75/rp49
F
NOS1/rp49
F'
NOS1/rp49
L5D2 L5D3 L5D4 L5D5 L5D6 L5D7 EW LW PP1 PP2
G
spo promoter relative luciferase activity
a a b ab a a b ab
EGFP OE + + − − − − − −
E75A OE − − + + − − − −
E75B OE − − − − + + − −
E75C OE − − − − − − + +
NO − + − + − + − +
H
E75B incomplete DBD
Physical interaction
E75A/C
ROREs
Halloween genes
(wandering)
Ecdysteriods biosynthesis
(prepupal)
NO
E75A/B/C C-teriminus
Physical interaction
HR3

Figure S5. Correlations among E75, HR3, NO and Nuclear receptor E75 isoforms mediate steroidogenesis autoregulation and regulate developmental timing during the larval-pupal transition in *Bombyx*

(A-C') Developmental profiles of *HR3* (A), *E75* (B), *NOS1* (C), and *NOS2* (C') mRNA levels in the prothoracic glands.

(D-F') Developmental profiles of *HR3* (D), *E75* (E), *NOS1* (F), and *NOS2* (F') mRNA levels in the fat body.

(G) HEK 293 cells were co-transfected with the *E75A/B/C* (*EGFP* as a control) expression constructs, the pGL3 basic plasmids containing ~2.5-kb promoter regions of *spo* and the *hsp70* basal promoter regulating the expression of firefly luciferase (Fluc), and a reference reporter plasmid carrying *Rellina* luciferase (Rluc). DETA-NO was added at 32 h after transfection and the dual luciferase assays were performed 16 h later. The luciferase activity fold change is defined as the relative luciferase activity induced by *E75A/B/C* overexpression compared to *EGFP* overexpression.

(H) E75A/C act as a transcription activator to induce Halloween gene expression and a transcriptional repressor to inhibit HR3 transactivation ability in promoting ecdysteroid biosynthesis and developmental transitions, and either function of E75 could be reversed by NO. Lacking a complete DBD, E75B does not act as an independent transcription activator, but antagonizes the transactivation ability of E75A/C; E75B serves as an equal transcriptional repressor for HR3. Acting independently or through HR3 inhibition, E75 isoforms function in a context-specific manner. The E75-mediated regulatory loop represents a fine autoregulation of steroidogenesis which contributes to the precise control of developmental timing.

20-Hydroxyecdysone (20E) Primary Response Gene *E75* Isoforms Mediate Steroidogenesis Autoregulation and Regulate Developmental Timing in *Bombyx*

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