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

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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). In insects, 20-hydroxyecdysone (20E; ecdyson 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
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).

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. 1D).

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. 1E). Moreover, Western blotting using EcR-B1, USP, and Br-C antibodies revealed a decrease in their protein levels in the E75 RNAi larvae (Fig. 1E’), 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. 2A), suggesting that E75 is required for maintaining ecdysteroid titers in Bombyx.
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 HPLC (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. 2C). 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 the prothoracic glands isolated from E75 RNAi larvae (34). Hormone production in endocrine organ cells, were misshaped in the prothoracic gland cells from the E75 RNAi larvae, supporting the reduced ecdysone production (Fig. 2C). Meanwhile, the mRNA levels of shd decreased by 80% in the fat body.
from the E75 RNAi larvae (Fig. 2D). 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 pHPLC 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. 2D). 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. 3D). 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. 3F). 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. 3F). In conclusion, overexpression of E75A/C up-regulates Halloween genes, promotes ecdysteroid biosynthesis, and accelerates metamorphosis, whereas E75B overexpression might have opposing effects.
**E75 Regulates Steroidogenesis**

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 ROREs 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 ROREs. 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 ROREs (except one in *shd*) when *E75A* was overexpressed, 12 ROREs (except one in *sad* and the other in *shd*) when *E75C* was overexpressed, but no ROREs when *E75B* was overexpressed (Fig. 4, K–O).

All the responsive 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. *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 ROREs in the promoter regions of all five Halloween genes and directly induces gene expression.

**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 µg; ~10⁶ 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.
**E75 Regulates Steroidogenesis**

![Diagram of luciferase assays](image)

**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.

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. 5E). Importantly, co-infection with E75B nearly blocked the ability of E75A/C to reduce body size (Fig. 5E). Seventy two h after BmNPV infection, ecdsysteroid titers significantly increased in the E75A/C-overexpressed larvae, and this increase was blocked by co-infection with E75B (Fig. 5F), suggesting that E75B antagonizes the transactivation ability of E75A/C.

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**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).
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 the amount of EGFP (E75C).

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.

E75 Regulates Steroidogenesis

CRISPR/Cas9-mediated genome editing is becoming a powerful tool for functional studies in *Bombbyx* (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, as shown in Figure 5G. These results suggest that E75B plays a crucial role in the regulation of steroidogenesis.
behavior and elevated ecdysteroid titer (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. 6A). By contrast, when E75B was co-transfected with E75A, E75B, or E75C, the two proteins frequently co-localized at some aggregating chromatin spots (Fig. 6B). 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. 6E). 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. 6F). When coE75A/B/C was co-transfected with E75A, E75B, or E75C, the two proteins always co-localized at the aggregating chromatin spots (Fig. 6G). Nevertheless, E75noN had no co-localization with E75A/C but co-localized with E75B (Fig. 6H). 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/C antagonized the transactivation ability of E75A/C in a dose-dependent manner (Fig. 6, I and J), 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 2A). These E75 RNAi silkworms exhibit phenotypic defects similar to Drosophila E75A and E75C null mutants (9).

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Second, E75A/C overexpression up-regulates Halloween gene expression. 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. 6A). By contrast, when E75B was co-transfected with E75A, E75B, or E75C, the two proteins frequently co-localized at some aggregating chromatin spots (Fig. 6B). 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. 6E). 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. 6F). When coE75A/B/C was co-transfected with E75A, E75B, or E75C, the two proteins always co-localized at the aggregating chromatin spots (Fig. 6G). Nevertheless, E75noN had no co-localization with E75A/C but co-localized with E75B (Fig. 6H). 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 J), 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
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, HA, 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, HA, 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 ~2.3-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 development.

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. S5H). 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. S5H).

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. S5G). Thus, the ability of E75A/C to promote ecdysteroid biosynthesis and developmental transition could be reversed by NO (supplemental Fig. S5H). 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). βtzt-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. S5H). 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...
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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 EGF 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 (25).

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), TAATAGCACTTACATAGGGCTT-AAGTGGCATGGTCGTTAGTATTAGCTTAAATAG-CAGTTAAAATAGGGTACGTCCGGTTATCACCCTGAA-AAAATGGCGCAGCGGCTGCTCTTTT, 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 pEx4-4 vector containing the BmNPV iel1 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, Gemany). 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.
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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, 43). For some experiments, two constructs of EGFPl, 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 pEx-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 E75nO), 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 pEx-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 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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