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. *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. 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. 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. 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.