The Steroid Hormone 20-Hydroxyecdysone Up-regulates Ste-20 Family Serine/Threonine Kinase Hippo to Induce Programmed Cell Death*

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Background: 20E and Hippo signaling promote PCD, although the interaction between them remains unclear.

Results: 20E up-regulates Hippo via EcRβ1, USP1, and HR3, and Hippo has negative feedback effects on HR3.

Conclusion: 20E up-regulates Hippo to induce PCD.

Significance: This study identifies 20E as a new input of the Hippo signaling pathway.

The steroid hormone 20-hydroxyecdysone (20E) and the serine/threonine Ste-20-like kinase Hippo signal promote programmed cell death (PCD) during development, although the interaction between them remains unclear. Here, we present evidence that 20E up-regulates Hippo to induce PCD during the metamorphic development of insects. We found that Hippo is involved in 20E-induced metamorphosis via promoting the phosphorylation and cytoplasmic retention of Yorkie (Yki), causing suppressed expression of the inhibitor of apoptosis (IAP), thereby releasing its inhibitory effect on caspase. Furthermore, we show that 20E induced the expression of Hippo at the transcriptional level through the ecdysone receptor (EcR), ultraspiracle protein (USP), and hormone receptor 3 (HR3). We also found that Hippo suppresses the binding of Yki complex to the HR3 promoter. In summary, 20E up-regulates the transcription of Hippo via EcRβ1, USP1, and HR3 to induce PCD, and Hippo has negative feedback effects on HR3 expression. These two signaling pathways coordinate PCD during insect metamorphosis.

Precisely controlled programmed cell death (PCD) is required by multicellular organisms during normal development. Extrinsic factors (i.e. hormones and growth factors) and organ-intrinsic factors coordinate PCD, yet the interactions between these two types of signal are poorly understood. Examples of well studied extrinsic factors are the small lipophilic hormones (including steroids, retinoic acid, and thyroid hormone), which direct the appropriate pattern of cell death. 20-Hydroxyecdysone (20E) is a novel steroid hormone that is being increasingly studied in the context of PCD.

Studies in Drosophila have provided our best understanding of the molecular mechanisms through which 20E triggers PCD. This pathway involves a two-step transcriptional cascade: first, 20E binds to the ecdysone receptor (EcR) and its heterodimeric partner ultraspiracle protein (USP) to form a transcription complex. The 20E-EcR-USP complex directly regulates primary response gene expression, including hormone receptor 3 (HR3) and Broad-Complex. Subsequently, several late second-response genes are induced, like the death activator genes. Finally, the death inducers trigger PCD by overcoming the inhibitory effect of the DIAP1 (Drosophila inhibitor of apoptosis protein 1) on caspases that execute apoptosis.

In addition to these extrinsic hormonal signals, organ-intrinsic mechanisms can coordinate PCD in response to external stimuli. Genetic studies in Drosophila defined a conserved Hippo signaling pathway that controls organ size by both restricting cell proliferation and promoting apoptosis. The serine/threonine Ste-20-like kinase Hippo represents a core component of the Hippo pathway that is activated via autophosphorylation, and in turn triggers a series of serine/threonine phosphorylation events that limit the activity of the transcriptional coactivator Yorkie (Yki) by cytoplasmic sequestration. Yki functions together with the TEAD/TEF family transcription factor Scalloped (Sd) to induce the expression of Hippo pathway-responsive genes.

Metamorphosis is a biological process by which holometabolous insects develop from larva into adults. During this process, the larval components of the insect are degraded by PCD. Metamorphosis is itself a complex process that is controlled by fluctuation of hormones. Thus, metamorphosis of insects provides a model to explore the interaction of extrinsic and intrinsic factors in the regulation of PCD. Here we used cotton bollworms (Helicoverpa armigera), a crop pest that belongs to...
Lepidoptera: Noctuidae, as a model to investigate the interaction between 20E and the Hippo signaling pathway during metamorphosis. By conducting the research, we may further understand the molecular mechanism of the two pathways that coordinate the PCD, and find the novel molecular targets to effectively control the pest.

We found that 20E up-regulates the transcription of Hippo via EcRBI, USP1, and HR3 to induce PCD. As an upstream regulator of the Hippo signaling pathway, HR3 is suppressed by Hippo through phosphorylating Yki and limiting its activity. These data identify 20E as a new input of the Hippo signaling pathway.

### Experimental Procedures

**Insect**—Cotton bollworms were fed with an artificial diet made from wheat and soybean at 27 ± 1 °C under 14:10 h light/dark cycles in our laboratory (14).

**RNA Interference (RNAi) of Hippo in the Larvae**—Double-strand RNA (dsRNA) was synthesized using the MEGA script RNAi Kit (Ambion, Austin, TX) according to the manufacturer’s instructions. The primers used to amplify the cDNA templates are listed in Table 1. For Hippo knockdown, ~3 μg of dsRNA was injected twice into the larvae at the 6th instar 12 and 72 h. Controls were injected with dsGFP. In addition, in the 20E induction group, the 6th instar 12-h larvae were fed with a diet containing 2 μM 20E continuously. Each treatment was performed on 30 larvae and was repeated three times for statistical accuracy. At the end of the experiment, total RNA was extracted for quantitative (q)RT-PCR analysis. Histologic sections for hematoxylin and eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay were performed as described by Liu et al. (15).

For EcRBI, USP1, and HR3 knockdown, 3 μg of dsRNA was injected into the larvae at the 6th instar 6 h, and the larvae were fed with diet containing 2 μM 20E continuously from 6th instar 30 h. At 6th instar 48 h, total RNA and protein were extracted for qRT-PCR and Western blot analysis, respectively.

**RNAi in the Epidermal Cell Line of H. armigera (HaEpi)**—The primers for dsRNA synthesis and qRT-PCR are listed in Table 1. Two micrograms of dsRNA was transfected with RNAfekt transfection reagent (Tiangen, Beijing, China) according to the methods described by Shao et al. (16).

**Overexpression in HaEpi Cell Line**—The open reading frame (ORF) of Hippo and Yki were amplified using corresponding primers (Table 1). These products were then inserted into the pEX-4-His or pEX-4-RFP-His (pEX-4-His vector fusing with RFP) vector. The inserts were verified by DNA sequencing. The plasmids were transfected into HaEpi cells with RNAfekt transfection reagent (Tiangen, Beijing, China), according to the manufacturer’s instructions and as described previously (17). The expression was confirmed by Western blot with anti-His antibody (Zhongshan Biotechnique, Beijing, China).

**Nuclear and Cytoplasmic Protein Extraction**—Twenty-four hours after transfection of dsHippo, Yki-RFP was overexpressed for 48 h. The cells were washed with Dulbecco’s phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄) and centrifuged. The cells were then suspended in buffer A (10 mM HEPES, 10 mM KCl, 0.2 mM EDTA, 3 mM MgCl₂, 1 mM 1,4-dithiothreitol (DTT), 1 mM PMSF, 1% mixture, and 1% Nonidet P-40) for 10–15 min on ice and centrifuged. The supernatant served as the cytoplasmic fraction and the cell pellet was then suspended in buffer B (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1% mixture). After vibration for 30 min, the cells were centrifuged at 16,000 × g, and the supernatant used as the nuclear fraction.

The clear separation of cytoplasmic and nuclear protein was proved by immunoblotting with antibody against the cytoplasmic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the nuclear protein histone-H3 antibody (ProteinTech Group, Chicago, IL).

**Preparation of Antiserum against Hippo**—Specific primers were designed to amplify the sequence coding for the serine/threonine protein kinases catalytic domain of Hippo (HipposTCK-expF and HipposTCK-expR, Table 1). The DNA fragments were ligated into expression vector pGEX-4T-1, and overexpressed in Escherichia coli Rosetta host cell. The transformant was cultured in Luria-Bertani broth (LB) containing 100 μg/ml of ampicillin and then induced for 4 h with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) after the cells grew to the optical density. Harvest cell pellets were suspended in phosphate-buffered saline (PB, 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) containing 0.2% Triton X-100 and were sonicated. The target protein exists primarily in the supernatant, which was then purified by glutathione-Sepharose 4B according to the manufacturer’s instructions.
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Two hundred μg of the purified protein was homogenized with the same volume of complete Freund’s adjuvant (Sigma) and then injected hypodermically into the back of a rabbit. The second injection was performed 3 weeks later with the emulsified mixture of protein and incomplete Freund’s adjuvant (Sigma). After 2 weeks, 500 μg of protein was injected into the back muscle of the rabbit. The specificity of the antiserum was determined by immunoblotting.

Immunoblotting—Isolated proteins were separated using 7.5% SDS-PAGE, and then transferred onto a nitrocellulose membrane. After blocking with 2% nonfat milk in TBS for 1 h, the membrane was incubated with the primary antibody (1:100 dilution in the blocking solution) overnight at 4 °C. After the membrane was washed with TBST (0.1% Tween 20 in TBS) for 3 × 10 min, the membrane was incubated with a secondary antibody conjugated with alkaline phosphatase (1:10,000) (Sangon, Shanghai, China) in the blocking buffer. The membrane was then washed in 3 times with TBST and in TBS for 10 min and visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride.

Dephosphorylation via λ-Protein Phosphatase Treatment—λ-Protein phosphatase treatment was performed according to the manufacturer’s specifications (Millipore, Temecula, CA). The fat body (0.5 g) of the 6th instar 96-h larvae was homogenized in 1 ml of TBS. The homogenate was incubated for 30 min with 500 units of λ-protein phosphatase and 25 μM DTT in reaction buffer at 37 °C. The sample was then prepared for immunoblotting.

qRT-PCR—Total RNA was extracted from tissues or the HaEpi cells using Unizol Reagent (Biostar, Shanghai, China). Five micrograms of RNA was reverse transcribed into cDNA (Moloney murine leukemia virus reverse transcriptase, BioTeke Corp., Beijing, China), and qRT-PCR was performed using 2× SYBR RT-PCR pre-mixture (BioTeke Corp.) with a CFX96™ real-time thermal cycler (Bio-Rad). β-Actin was amplified for internal standardization. The primers for qRT-PCR are listed in Table 1.

Detection of Caspase-3/7 Activity—Activity of caspase-3/7 was detected using CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen). 2 μM Caspase-3/7 Green Detection Reagent was added to the medium and incubated at 37 °C for 1 h. The following steps were performed according to the manufacturer’s specifications. The nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg/ml in water) for 10 min. Fluorescence was detected with a Laser Scan Confocal Microscope Carl Zeiss LSM 700 (Thornwood, NY).

Hormone Regulation of Hippo Transcription—Normal HaEpi cells were treated with 2 μM 20E. Total RNA was extracted from the cells at time intervals of 1 to 12 h for the synthesis of cDNA. qRT-PCR was used to detect the hormone effects on the transcription of Hippo.

Electrophoretic Mobility Shift Assay (EMSA)—For our EMSA, pIEx-Yki-RFP was co-transfected with pIEx-Hippo into HaEpi cells for 48 h, or transfected into HaEpi cells for 48 h after Hippo was knocked down. The nuclear extracts were used for EMSA. The digoxigenin (Dig)-labeled probes used in the EMSA were designed according to the Sd binding sites as shown in Fig. 5C (produced by the Sangon Company, Shanghai, China). Ten micrograms of nuclear extracts in binding buffer (Beyotime Institute of Biotechnology, Nantong, China) was incubated with 100 fmol of Dig-labeled probe. A 50-fold amount of unlabeled probe was preincubated with the nuclear protein for 10 min before the addition of the Dig-labeled probe in competition experiments. In the supershift assays, antibody was incubated with the reaction mixture on ice for 1 h before loading on the gel. The mixture was run on a 6.5% polyacrylamide gel at 10 V/cm and then transferred onto a nylon membrane (Immobilon-Ny; Millipore, Milford, MA). After being blocked for 30 min with 1% (w/v) blocking reagent (Roche Applied Science, Mannheim, Germany), an anti-Dig-phosphatase antibody (Roche Applied Science) was used to detect the probe, which was then visualized with bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride.

Chromatin Immunoprecipitation (ChIP) Assay—For the ChIP assay, pIEx-Yki-RFP was co-transfected with pIEx-Hippo into HaEpi cells for 48 h, or transfected into HaEpi cells for 48 h after Hippo was knocked down. A ChIP assay used the antibody against RFP (Cwbio, Beijjing, China) was performed as described by Liu et al. (20). The DNA was purified and used as the template for qRT-PCR to detect the Sd binding sites containing the probe4 region in the HR3 promoter with the HR3SD-ChIPF and HR3SD-ChIPR primers (Table 1). The coding DNA sequence region (HR3-CD5) that amplified by HR3-qRTF1 and HR3-qRTRI was used as control.

Results

Knockdown of Hippo Delays 20E-induced Metamorphosis by Inhibiting Programmed Cell Death—We identified Hippo in H. armigera through sequence analysis, alignment, and phylogenetic analyses. These analyses showed that Hippo is a phylogenetically conserved protein, which contain a serine/threonine protein kinases, catalytic domain (aa 29 to 280), and a C-terminal SARAH (Sav-RASSF-Hpo domain) domain of Mst1 (Mammalian STE20-like protein kinase) (aa 439 to 487). To investigate whether Hippo was involved in 20E-induced metamorphosis, Hippo was depleted by injecting dsRNA into the 6th instar control larvae and 20E-induced larvae. As Fig. 1A shows, dsHippo injection delayed pupation for 19 h compared with the dsGFP-injected larvae. Treatment with 20E promoted pupation, which occurred 21 h earlier than larvae treated with dimethyl sulfoxide. However, combined treatment with dsHippo and 20E delayed pupation for 24 h compared with dsGFP and 20E treated controls. These data suggest that Hippo functions in 20E-regulated metamorphosis.

To determine how Hippo participates in 20E-induced metamorphosis, the midgut and fat body of larvae that presented a phenotype of delayed development were dissected at the wandering stage and stained with hematoxylin and eosin. As shown in Fig. 1B, the midgut of the larvae injected with dsGFP separate from the imaginal midgut, and the fat body of the dsGFP-injected control group began to undergo histolysis. By contrast, the larval midgut in the dsHippo-injected larvae could not separate from the imaginal midgut, and the fat body cells maintained an intact structure. Furthermore, in situ PCD was detected by TUNEL assay. Few TUNEL signals were observed.
after Hippo was knocked down. These results imply that Hippo is involved in 20E-induced metamorphosis via promoting PCD.

**Hippo Promotes the Phosphorylation and Cytoplasmic Retention of Yki to Regulate PCD-related Genes**—In the Hippo signaling pathway, the upstream components converge to a common factor: the transcriptional coactivator Yki (18). Thus, the phosphorylation status and localization of Yki can be used as readout for the Hippo signaling pathway activity. According to Fig. 1A, silence of Yki decreased the delays caused by Hippo knockdown, which indicates that Hippo regulates PCD through Yki.

To investigate how Hippo regulates PCD, we detected the effects of Hippo on Yki. Our Western blot analysis shows that after Hippo expression was silenced in HaEpi cells, Yki phosphorylation (visualized as a shift in Yki mobility), and the cytoplasmic location of Yki were suppressed (Fig. 2A). The purity of cytoplasmic and nuclear protein was assessed by detection of the cytoplasmic protein marker GAPDH and the nuclear protein histone-H3. A Coomassie Blue-stained gel was used as loading control. Nu, nuclear fraction; Cy, cytoplasm fraction. B, the dsRNA of Hippo or GFP was transfected into HaEpi cells for 48 h. The relative expression levels of Hippo and IAP are shown after Hippo knockdown in HaEpi cells. The results were based on the 2−ΔΔCT method in the presence of normalized β-actin gene. Student’s t test; *, p < 0.05; **, p < 0.01. C, representative images showing caspase 3/7 activity in Hippo-RFP or RFP overexpressing cells are presented. Red fluorescence indicates the expression of Hippo-RFP or RFP. Green fluorescence indicates activity of caspase 3/7. Blue fluorescence indicates cell nuclei stained with DAPI. Scale bar = 20 μm.
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**FIGURE 3. Hippo is highly expressed during molting and in metamorphically committed stages.** A, the relative expression of Hippo is shown in different tissues at three stages of metamorphosis: feeding (F), molting (M), and wandering (W). B, the relative expression of Hippo is shown at multiple developmental stages in the midgut and fat body. 5F, 5th instar feeding stage; 5M, 5th instar molting stage; WH, white head; the 6 – 0 to 6 – 120 are larvae from 6th instar 0 h to 6th instar 120 h after ecdysis; p0, pupa on day 0. The results were based on the 2−ΔΔCT method in the presence of normalized β-actin gene. Asterisks indicate significant differences (Student’s t test; *, p < 0.05; **, p < 0.01). C, Western blot detecting the specificity of the antibody against Hippo with the midgut homogenates from 5th instar feeding larvae in 10% SDS-PAGE. D, the protein level of Hippo during development in the midgut and fat body was detected by Western blot using anti-Hippo antibody. E, the fat body sample from the 6th instar 96-h larvae was extracted and treated with λ-protein phosphatase (λAPP) to determine the Hippo phosphorylation. Hippo-P, phosphorylated Hippo.

expression, the key factors of the 20E signaling pathway were separately knocked down in HaEpi cells. The qRT-PCR results showed that Hippo transcription could not be induced after knockdown of EcRB1 (Fig. 4B), USP1 (Fig. 4C), or HR3 (Fig. 4D). We then tested the transcription level of Hippo in the 20E-treated midgut of larvae after EcRB1 (Fig. 4E), USP1 (Fig. 4F), or HR3 (Fig. 4G) was knockdown, respectively. The qRT-PCR data are correlated to the results in vivo. In addition, we further verified the effects of EcRB1, USP1, and HR3 on Hippo at protein levels in vitro. The Western blot analysis showed that the induction of 20E to Hippo was blocked after knockdown of EcRB1, USP1, or HR3 (Fig. 4H), which is in accord with the results of qRT-PCR. These results showed that the induction of Hippo by 20E is mediated by EcRB1, USP1, and HR3.

Hippo Signaling Regulates the Transcription of HR3—Feed-back regulation of upstream pathway components is a common feature of many signaling pathways. Because previous experiments placed EcRB1, USP1, and HR3 upstream of Hippo, we wished to test whether this is also the case for these three transcriptional factors. To this end, we knocked down Hippo by RNAi in HaEpi cells to detect the transcription of EcRB1, USP, and HR3. The qRT-PCR results indicated that the transcript level of HR3 was increased when Hippo was knocked down, whereas the transcription of EcRB1 and USP1 were not affected (Fig. 5A). These data imply that Hippo negatively regulates the expression of HR3.

To further validate the conclusion, immunoblotting was used to investigate the effects of knockdown of Hippo on the RFP expression driven by HR3 promoter. The pIEx-HR3pro-RFP plasmid was constructed in our previous work (20). The original hr5 enhancer and IE promoter in the pIEx-4 plasmid were replaced by a 1084-bp DNA fragment from the 5′ upstream region of H. armigera HR3. The Western blot assay

sion of IAP, and releasing the inhibitory effect of IAP on the caspases.

**Hippo Is Highly Expressed during Molting and the Metamorphically Committed Stage**—To further explore the role of Hippo in 20E-induced metamorphosis, we examined the spatio-temporal expression pattern of Hippo. According to our qRT-PCR analysis, Hippo mRNA was detected in the integument, midgut, fat body, and hemocytes (Fig. 3A). The developmental expression profile of Hippo in the fat body and midgut from the 5th instar larvae to the pupae was further examined. As we can see from the line graph, the mRNA expression level of Hippo showed an upward trend during molting and the wandering stage (Fig. 3B). At protein levels, Hippo had similar developmental change profile, with higher expression during molting and metamorphically committed stage (Fig. 3D). In addition, two bands were detected during metamorphic molting. After the fat body protein sample from the 6th instar 96-h larvae was treated with λ-protein phosphatase, the upper protein band degraded to the lower band (Fig. 3E), thereby indicating that the high molecular weight band is the phosphorylated form of Hippo. These results indicate that Hippo is highly expressed during molting and when the larvae are committed to metamorphosis.

20E Up-regulates the Transcription of Hippo—Peak expression of Hippo occurs when hemolymph levels of 20E are high (22). Thus, we measured the transcript level of Hippo in 20E-treated HaEpi cells to investigate the link between Hippo expression and 20E. Our qRT-PCR analysis revealed that Hippo mRNA shows an upward trend from 12 to 24 h after 20E induction (Fig. 4A), indicating that Hippo can be induced by 20E.

To determine the exact regulatory mechanism of 20E on Hippo expression, the key factors of the 20E signaling pathway were separately knocked down in HaEpi cells. The qRT-PCR results showed that Hippo transcription could not be induced after knockdown of EcRB1 (Fig. 4B), USP1 (Fig. 4C), or HR3 (Fig. 4D). We then tested the transcript level of Hippo in the 20E-treated midgut of larvae after EcRB1 (Fig. 4E), USP1 (Fig. 4F), or HR3 (Fig. 4G) was knockdown, respectively. The qRT-PCR data are correlated to the results in vivo. In addition, we further verified the effects of EcRB1, USP1, and HR3 on Hippo at protein levels in vitro. The Western blot analysis showed that the induction of 20E to Hippo was blocked after knockdown of EcRB1, USP1, or HR3 (Fig. 4H), which is in accord with the results of qRT-PCR. These results showed that the induction of Hippo by 20E is mediated by EcRB1, USP1, and HR3.
showed that the silence of Hippo increased the HR3 promoter-activated RFP expression (Fig. 5B), which are in accord with the results of qRT-PCR.

The observation that HR3 expression is regulated by Hippo raises the question of whether the effects are direct or indirect. Considering that Yki partners with DNA-binding transcription factors, such as Sd, binds to the regulation element of the target gene, we searched for the Sd binding sequence (19) in the 5′ upstream region of the H. armigera HR3 (20). A total of five sites that match the Sd binding consensus sequence were found (Fig. 5C). To determine whether Yki and the transcription factor complex physically interact with the HR3 promoter, we carried out EMSA experiments using HaEpi cells expressing Yki-RFP. The shift bands were only observed in samples incubated with the Dig-labeled probe4 (Fig. 5D). Given that Hippo could regulate the phosphorylation and localization of Yki, we overexpressed or knocked down Hippo in HaEpi cells to investigate the effect of Hippo on the binding ability of Yki. EMSA showed that overexpressing Hippo weakened the shift band, whereas Hippo knockdown resulted in increased binding (Fig. 5D). We showed that the protein-DNA interaction was specific, as it was competed away by unlabeled probe. The interaction disappears after the consensus sequence of probe4 was mutant (Fig. 5D). Using ChIP, we further validated the results of EMSA. According to the bar chart, after Yki-RFP was overexpressed, binding of the Yki-transcription factor complex to the promoter of HR3 was increased by 5.4-fold as compared with the RFP control. The percentage of input chromatin dipped to 6.3% after Hippo was overexpressed. However, the Hippo knockdown increased the percentage to 12.9% (Fig. 5E). These data indicate that HR3 is a direct transcriptional target of the Yki, and that Hippo suppresses the binding of Yki complex to the HR3 promoter by regulating Yki phosphorylation.

Discussion

Previous studies have revealed that both 20E and Hippo signaling regulate PCD. This study provides evidence that the 20E and Hippo signaling pathways are interlinked, and influence PCD during insect metamorphosis.

20E Induces PCD in Part via the Hippo Signaling Pathway—It has previously been shown that 20E-induced death activator expression overcomes the suppression of DIAP1 on caspase and triggers cell death (7). Mechanistically, the autophosphorylation of Hippo initiates the core kinase cascade and leads to the phosphorylation and cytoplasmic sequestration of Yki, consequently decreasing expression of DIAP1 (21). In this article, we present evidence linking the two signaling pathways that regulate PCD. Our data shows that knockdown of Hippo decreases the promotion of 20E-induced metamorphosis, supporting that Hippo is involved in the PCD regulated by 20E. Consistent with the quintessential theory, we also found that Hippo induces Yki phosphorylation and cytoplasmic sequestration, and that the over-expression of Hippo can activate the action of caspases in HaEpi cells. These data infer that 20E induces PCD partly via the Hippo signaling pathway, in which...
Hippo suppresses the transcriptional activation of Yki on IAP (Fig. 6).

20E Is an Input of Hippo Signaling Pathway—Hippo appears to be a central signaling hub capable of integrating numerous inputs. Several signaling modules at the cell membrane were identified as Hippo regulators, including FERM domain proteins Merlin and Expanded, the protocadherins Fat and Dachsous, the WW and C2 domain-containing protein Kibra, and the apical transmembrane protein Crumbs (18). Whether Hippo signaling is responsive to other cellular inputs is an open question. Our developmental profile studies reveal that the expression level of Hippo was elevated during molting and pupation, when 20E titers were high (22). This data stimulated us to further study whether 20E regulates the function of Hippo. Furthermore, we found that 20E up-regulates the transcription of Hippo via EcR-B1, USP1, and HR3. Taken together,
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PI3K signaling and Yki activity (29). This common negative feedback loop may play important roles in maintaining steady-state levels of signaling strength (30).

In summary, the steroid hormone 20E up-regulates the transcription of Hippo via EcRB1, USP1, and HR3, and Hippo has negative feedback effects on the expression of HR3. Taken together, we believe these two signaling pathways coordinate PCD during insect metamorphosis (Fig. 6).

**Author Contributions**—D.-J. D. designed, performed, and analyzed the experiments in this manuscript. Y.-P. J. and W. L. performed the overexpression and RNAi in HaEpi cells. J.-X. W. participated in the design and coordination of the work. X.-F. Z. conceived the study and helped to draft the final version of this manuscript. All authors reviewed the results and approved the final version of the manuscript.

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**Hippo Has Negative Feedback Regulation on Transcription of HR3**—Loss of Hippo or increased Yki activity can drive the expression of genes encoding upstream regulators of the Hippo pathway, such as Kibra (26), Ex (27), and Fj (28). Similarly, feedback regulation of upstream pathway components appears to be a shared feature of many signaling pathways. This is consistent with our observation that 20E signaling positively regulates Hippo transcription, and that activation of Hippo leads to inhibition of HR3 via phosphorylating and inactivating Yki. In support of our conclusions, a similar interaction was shown for...
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