20-hydroxyecdysone activates Forkhead box O to promote proteolysis during *Helicoverpa armigera* molting

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SUMMARY STATEMENT

The present study used an insect as a model to reveal that steroid hormone 20E upregulates PTEN expression, which inhibits insulin-induced Akt and FoxO phosphorylation, resulting in non-phosphorylated-FoxO nuclear localization.

ABSTRACT

Insulin inhibits transcription factor forkhead box O (FoxO) activity, and the steroid hormone 20-hydroxyecdysone (20E) activates FoxO; however, the mechanism is unclear. We hypothesized that 20E upregulates phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) expression to activate FoxO, thereby promoting proteolysis during molting in the lepidopteran insect *Helicoverpa armigera*. FoxO expression is increased during molting and metamorphosis. The knockdown of *FoxO* in fifth instar larvae results in larval molting failure. 20E induces FoxO non-phosphorylation and nuclear translocation. Insulin, via Akt, induces FoxO phosphorylation and cytoplasm localization. 20E represses insulin-induced Akt phosphorylation and FoxO phosphorylation. 20E, via ecdysone receptor B1 (EcRB1) and the ultraspiracle protein (USP1), upregulates PTEN expression, which represses Akt phosphorylation, thereby repressing FoxO phosphorylation. The non-phosphorylated FoxO enters the nucleus and attaches to a FoxO binding element in the upstream region of the Broad isoform 7 (*BrZ7*) gene to regulate *BrZ7* transcription under 20E induction. 20E upregulates *FoxO* expression via EcRB1 and USP1. FoxO regulation of *BrZ7* expression regulates CarboxypeptidaseA expression for final proteolysis during insect molting. Hence, 20E activates FoxO via upregulating PTEN expression to counteract insulin activity and promote proteolysis.

Keywords: Forkhead box O, Protein kinase B/Akt, PTEN, Steroid hormone, Proteolysis.

INTRODUCTION

Forkhead box O (FoxO) proteins are a subgroup of the Forkhead transcription factor family (Kaufmann and Knochel, 1996). Mammals have four *FoxO* genes: *FoxO1*, *FoxO3*, *FoxO4*, and *FoxO6* (Furuyama et al., 2002). However, only one *FoxO* (*dFoxO*) gene was identified in *Drosophila* (Junger et al., 2003). FoxO has different functions in various cellular processes (Eijkelenboom and Burgering, 2013), including orchestrating the expression of genes involved in apoptosis in rat sympathetic neurons (Gilley et al., 2003), cellular differentiation in *Drosophila* (Puig and Mattila, 2011), and autophagy in skeletal muscles of mice (Mammucari et al., 2007). FoxO3 stimulates protein degradation in mouse C2C12 myotubes (Zhao et al., 2007). DAF-16, a FoxO in *Caenorhabditis elegans*, promotes proteasomal degradation (Vilchez et al., 2012). FoxO activity is repressed by the insulin pathway (Barthel et al., 2005). In mammals, FoxO is phosphorylated by the phosphorylated-protein kinase B (PKB, also known as Akt) and maintained in the cytoplasm under insulin regulation (Saltiel and Kahn, 2001), which inhibits FoxO transcriptional activity in the nucleus (Matsuzaki et al., 2003). When the insulin pathway is blocked, FoxO is not phosphorylated and is translocated to the nucleus to initiate gene transcription, thereby inhibiting cell proliferation and promoting cell quiescence and apoptosis (Zhang et al., 2011). Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, also named phosphatase, tensin homologue/mutated in multiple advanced cancers (PTEN/MMAC1) is a tumor suppressor (Ali et al., 1999) and a negative regulator of insulin signaling, which inhibits Akt phosphorylation (Maehama and Dixon, 1998).

FoxO expression is upregulated by steroid hormone 20-hydroxyecdysone (20E), and is located in the cytoplasm during insulin induction in the lepidopteran insect *Helicoverpa armigera* (Hou et al., 2012). Feeding larvae with 20E causes nuclear localization of dFoxO in the fat body cells of *Drosophila melanogaster* (Colombani et al., 2005). 20E induces high expression and nuclear localization of FoxO, which upregulates the expression of brummer and acid lipase-1, as well promoting lipolysis in fat body cells during molting and pupation of *Bombyx mori* (Hossain et al., 2013). The lack of a juvenile hormone (JH)-producing organ, the corpora allata, elevates FoxO activity and results in small pupae (Mirth et al., 2014). Higher concentrations of 20E repressed insulin-induced gene expression in *H. armigera* (Liu et al., 2015). FoxO interacts with USP to mediate ecdysone biosynthesis (Koyama et al., 2014). Although these pieces of evidence suggested that 20E activates FoxO for molting and metamorphosis, the mechanism remains unclear.

Insect molting is a typical process to produce a new exoskeleton and shed the old cuticle during larva-to-larva, larva-to-pupa, or larva-to-adult development. Molting includes two processes: apolysis – the degradation of the old cuticle by proteases and chitinases in the molting fluid, secreted by the epidermis; and ecdysis – shedding of the old cuticle (Dubrovsky, 2005). Insect molting is triggered by a higher titer of 20E (Riddiford, 2003). 20E binds to its nuclear receptor, EcR, and forms a transcription complex with the heterodimeric ultraspiracle protein (USP). The complex then binds to the ecdysone response element (EcRE) to promote 20E-responsive gene transcription (Fahrbach et al., 2012; Riddiford et al., 2001). One of the 20E-response genes is the transcription factor Broad (*Br*), or Br-complex (*Br*-*C*), which is critical to initiate metamorphosis in the 20E pathway (Zhou and Riddiford, 2002). A newly identified Br isoform 7 (*BrZ7*) increases expression during metamorphosis and regulates metamorphosis in *H. armigera* (Cai et al., 2014b). Carboxypeptidase A (*CPA*), another 20E-response gene, is secreted into the molting fluid by the epidermis under 20E induction to degrade old cuticle proteins during apolysis in *B. mori* (Ote et al., 2005) and *H. armigera* (Sui et al., 2009). However, the mechanism by which 20E regulates the expression of Br and the cascade of the aforementioned genes expressions remains unclear.

The present study aimed to determine the mechanism of 20E's effects on FoxO activity. We revealed that FoxO expression increases during molting and metamorphosis in *H. armigera* under 20E regulation. *FoxO* knockdown, by injecting double-stranded RNA (dsRNA) into larvae, blocked larval molting and 20E-responsive gene expression. 20E upregulates PTEN expression and represses insulin-induced Akt phosphorylation, which inhibits insulin-induced FoxO phosphorylation, resulting in FoxO nuclear localization. In the nucleus, FoxO binds to the FoxO binding element (FoxOBE) at the 5′ upstream region of *BrZ7*. FoxO directly regulates *BrZ7* transcription, which promotes CPA expression for apolysis during molting under 20E induction. Accordingly, FoxO is a critical regulator in 20E-induced proteolysis during molting.

RESULTS

Conserved Forkhead box of FoxO

We identified *FoxO* from transcriptome sequencing of a cDNA from an epidermal cell line from *H. armigera* (HaEpi). The open reading frame of *FoxO* comprises 1,551 bp (GenBank accession number: KM008744). *FoxO* encodes a protein of 516 amino acids with a calculated molecular mass of 55.8 kDa. Three potential specific Akt phosphorylation sites (Thr-49, Ser-191, and Ser-255) were identified in FoxO. The DNA-binding domain, termed the “Forkhead box”, is located near the N-terminal region (94-183 aa) (Supplementary files: Fig. S1). *H. armigera* FoxO shares 85%, 77%, 47% and 36% similarities with *B. mori*, *Danaus plexippus*, *Drosophila melanogaster* and *Homo sapiens* FoxO, respectively (Supplementary files: Fig. S2).

20E induces FoxO expression during molting and metamorphosis

We examined the expression profile of FoxO in the epidermis, midgut, and fat body to determine the involvement of FoxO in 20E-induced molting and metamorphosis. The protein levels of FoxO in these tissues appeared to increase during the fifth molting stage (5M) or metamorphic stages (6th-72 h, 6th-96 h, and 6th-120 h), compared with the feeding stages (5F, 6th-24 h and 6th-48 h) (Figs. 1A and 1B). Given that the 20E titer is higher during molting and metamorphosis in the lepidopteran insect *Manduca sexta* (Riddiford, 2003), we suspected that FoxO expression was upregulated by 20E during molting and metamorphosis. To validate this hypothesis, we injected 20E into sixth instar 6 h larvae and observed an increased *FoxO* transcripts; juvenile hormone III (JH III) did not induce *FoxO* (Fig. 1C). Therefore, FoxO is likely involved in molting and metamorphosis via 20E induction.

FoxO knockdown blocks larval molting and gene expression in the 20E pathway

To examine the function of FoxO in molting and metamorphosis, we injected the dsRNA of *FoxO* into fifth instar 6 h larvae to knockdown FoxO expression. Western blotting and quantitative real-time reverse transcription PCR (qRT-PCR) showed that the protein and mRNA expressions of FoxO were successfully knocked down in the epidermis. Meanwhile, the expression of 20E nuclear receptor *EcRB1* and *USP1* were unaffected by *FoxO* knockdown. However, the expressions of transcription factor *BrZ7* and *CPA* were significantly inhibited (Fig. 2A and B). In the *FoxO* knockdown larvae, molting was obviously blocked (Fig. 2C). Statistical analysis showed that 62% of the larvae failed to shed their old cuticle to enter the sixth instar stage and eventually died (Fig. 2D) when *FoxO* was silenced. The results indicated that FoxO plays a critical role in molting by regulating BrZ7 and CPA expressions downstream of EcRB1 and USP1 in the 20E pathway.

When *FoxO* was knocked down, apolysis could not occur and the old cuticle could not be separated from the epidermis, unlike in the *dsGFP*-injected control. Moreover, the CPA protein was detected in the old cuticle and the epidermis in the *dsGFP* control, but not in the epidermis in *dsFoxO*-injected larvae (Fig. 3). These results suggested that FoxO regulates CPA expression to perform proteolysis during apolysis.

20E represses FoxO phosphorylation and regulates FoxO nuclear localization in HaEpi cells

Immunohistochemistry experiments were performed to investigate the subcellular localization of FoxO in the epidermis. FoxO was detected both in the cytoplasm and the nucleus in the epidermis, with increased levels in the nucleus during the fifth molting stage (5M) and the metamorphic stages (6th–72 h), when the old cuticle was separated from the epidermis, compared with fifth feeding stage (5F) (Fig. 4A). Western blotting further showed two FoxO immunoreactive bands, with the upper band being dominant at 5F. Treatment with lambda protein phosphatase (λPP) (5F + λPP) caused the upper band to almost disappear, with a concomitant increase in the lower band. These results suggested that the upper band is the phosphorylated form of FoxO. By contrast, two bands were attributed to FoxO at 5M and 6th-72 h, with the lower band being dominant (Fig. 4B). In addition, the phosphorylated FoxO was distributed in the cytosol, whereas the non-phosphorylated-FoxO was distributed in the nucleus (Fig. 4C and D). These results revealed that FoxO is mainly phosphorylated and located in the cytoplasm during the feeding stage, and is mainly non-phosphorylated and located in the nucleus during molting and metamorphic stages.

To confirm that 20E inhibits FoxO phosphorylation and induces FoxO nuclear localization, we incubated HaEpi cells with 20E and analyzed the subcellular localization of FoxO by immunocytochemistry. In the DMSO-treated control, FoxO was localized in the entire cell, including in the cytoplasm and the nucleus. FoxO showed increased nuclear localization after 6 h of incubation with 20E (Fig. 5A). Western blotting confirmed the increase in non-phosphorylated FoxO in the nucleus after 20E incubation (Fig. 5B and C). To confirm the 20E-induced nuclear localization of FoxO, the cells were cultured in Grace’s medium without FBS (starvation conditions) for further experiments. Under starvation conditions, FoxO was mainly localized in the nucleus, and insulin addition shifted the subcellular localization of FoxO from the nucleus to the cytoplasm. However, FoxO showed an increasingly nuclear localization after adding 20E (Fig. 5D). These results revealed that 20E inhibits FoxO phosphorylation and induces FoxO nuclear localization.

20E represses Akt phosphorylation to depress FoxO phosphorylation

To reveal the mechanism of 20E’s inhibition of FoxO phosphorylation, we analyzed the involvement of Akt in insulin-induced FoxO phosphorylation in HaEpi cells, because insulin, via Akt, regulates phosphorylation of FoxO in mammals (Saltiel and Kahn, 2001). Western blotting showed that insulin induced the phosphorylation of FoxO in 15 min (Fig. 6A). However, when *Akt* was knocked down, the insulin-induced phosphorylation of FoxO was significantly inhibited (Fig. 6B). These results confirmed that insulin, via Akt, induces FoxO phosphorylation in *H. armigera*. When insulin induced FoxO phosphorylation, Akt phosphorylation was also induced; in turn, when 20E inhibited the insulin-induced FoxO phosphorylation, Akt phosphorylation was also inhibited (Fig. 6C and D). These results suggested that 20E represses insulin-induced Akt phosphorylation, which inhibits insulin-induced FoxO phosphorylation.

20E inhibits Akt phosphorylation via upregulating PTEN expression

We examined the involvement of PTEN in 20E-induced repression of Akt phosphorylation. Akt phosphorylation was decreased and PTEN expression was increased during metamorphic molting (Fig. 7A). The expression of PTEN was upregulated by 20E induction in 3 h, at which point Akt was not phosphorylated. The protein synthesis inhibitor cycloheximide did not repress the 20E-induced PTEN expression at the protein or mRNA level, suggesting the direct regulation of 20E on PTEN expression (Fig. 7B). Knockdown of *EcRB1* or *USP1* significantly decreased the 20E-induced *PTEN* expression, by qRT-PCR analysis. The results confirmed that 20E upregulates *PTEN* expression via EcRB1 and USP1 (Fig. 7C). In the *dsGFP* control, 20E induced PTEN expression, and repressed insulin-induced Akt phosphorylation. In contrast, when *PTEN* was knocked down, 20E could not inhibit insulin-induced Akt phosphorylation (Fig. 7D). These data confirmed that 20E inhibits Akt phosphorylation via increasing PTEN expression.

FoxO directly regulates the transcription of BrZ7 during the 20E induction

We found a FoxOBE motif (5′-T**TGTTT**AA-3′, between -845 ~ -838 bp before the ATG) in the 5′ upstream region of *BrZ7* (Supplementary files: Fig. S3); however, the “C” in the conserved FoxOBE 5′-TTGTTTAC-3′ (Eijkelenboom and Burgering, 2013) was replaced by “A” in FoxOBE of *BrZ7*. Nevertheless, the highly conserved FoxOBE common core sequence (5′**-AAACA-**3′) in the complementary chain (Barthel et al., 2005) was retained. Given that the expression of *BrZ7* was suppressed after *FoxO* knockdown in the larval experiments, we examined the regulation by FoxO on the transcription of *BrZ7*. FoxO-GFP-His was overexpressed in HaEpi cells by transfection of plasmid pIEx-4-FoxO-GFP-His. Through a chromatin immunoprecipitation (ChIP) assay, a small amount of the quantitative real-time reverse transcription (qRT-PCR) product of the FoxOBE-containing DNA fragment was detected from the immunoprecipitates of the pIEx-4-GFP-His-transfected (empty vector) control samples after DMSO, JH, or 20E treatment, using primers BrZ7PF/PR. By contrast, large amounts of the FoxOBE-containing DNA fragment were obtained from the immunoprecipitates in the 20E-induced pIEx-4-FoxO-GFP-His-transfected cells, but not in the DMSO- or JH III-incubated cells (Fig. 8A). To exclude the non-specific binding of FoxO to DNA, we used qRT-PCR to examine the possibility that FoxO binds to other DNA fragments of *BrZ7*, using primers BrZ7F/R (150 bp, without intron), which were located 838

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bp downstream from the FoxOBE-containing DNA fragment. The data showed that FoxO did not bind to this fragment (Fig. 8B). The result showed that FoxO specifically binds to the *BrZ7* proximal promoter region during 20E induction.

FoxO isolated from DMSO-treated cells was phosphorylated, but FoxO isolated from 20E-induced cells was not phosphorylated (Fig. 8C). To confirm that the non-phosphorylated-FoxO directly bound to FoxOBE, electrophoretic mobility shift assays (EMSAs) were performed with a digoxigenin (Dig)-labeled-FoxOBE probe (5′-TTTTTGCA**TTGTTT**AAAATAGCAGC-3′) and the purified FoxO-GFP-His protein from the cells after DMSO or hormone induction. The FoxO-GFP-His from DMSO-treated cells did not shift the probe. However, a distinct shifted band was detected using the FoxO-GFP-His from the 20E-induced cells. Binding decreased when an unlabeled-FoxOBE probe was used as a competitive inhibitor. Anti-His antibodies that recognized FoxO-GFP-His produced a supershifted band. However, adding an anti-GST antibody (negative control) did not produce a supershifted band (Fig. 8D). These data suggested that the non-phosphorylated-FoxO binds directly to FoxOBE.

To confirm the specific binding of FoxOBE, a mutated, unlabeled-FoxOBE probe (FoxOBE-M, 5′-TTTTTGCA**ATGCATGC**AATAGCAGC-3′) was used as another competitive inhibitor. Binding of FoxO to the digoxigenin-labeled, FoxOBE probe was detected, which competed with the unlabeled-FoxOBE, but not with the unlabeled-FoxOBE mutated probe (Fig. 8E and F). These results indicated that FoxO binds to the FoxOBE of *BrZ7* to regulate BrZ7 transcription directly.

20E regulates the cascade of gene expression

To address the cascade of gene expression that occurs during 20E induction, *EcRB1*, *USP1*, *FoxO*, and *BrZ7* were knocked down separately. Knockdown of *EcRB1* or *USP1* in HaEpi cells repressed the 20E-induced expressions of *FoxO*, *BrZ7* and *CPA* (Figs. 9A and 9B). *FoxO* knockdown repressed *BrZ7* and *CPA* expressions, but did not repress *EcRB1* and *USP1* expression (Fig. 9C). *BrZ7* knockdown repressed *CPA* expression, but did not repress *FoxO* expression (Fig. 9D). These results identified the cascade of gene expressions during 20E induction. That is, 20E via EcRB1 and USP1, upregulates FoxO expression, which in turn directs BrZ7 expression, leading to CPA expression regulation.

DISCUSSION

The insulin pathway plays an important role in the regulation of cell size, cell number and critical body size (Gao et al., 2000). Insulin stimulates growth of the prothoracic gland (PG) to synthesize large amounts of 20E to initiate larval molting or metamorphosis (Mirth et al., 2005). 20E causes nuclear localization of dFoxO in the fat body cells of *D. melanogaster* to counteract insulin activity (Colombani et al., 2005). However, the mechanism of 20E counteracting insulin activity is not well known. Br or Br-C is a metamorphic inducer in the 20E pathway (Erezyilmaz et al., 2006). However, the mechanism by which 20E upregulates Br expression has not yet been determined. The present study revealed that 20E upregulates PTEN and FoxO expression, and inhibits Akt and FoxO phosphorylation, resulting in non-phosphorylated-FoxO nuclear localization. In the nucleus, FoxO binds to FoxOBE at the 5′ upstream region of *BrZ7* to promote *BrZ7* transcription, allowing BrZ7 to regulate CPA expression for protein degradation in apolysis during molting.

20E antagonizes the insulin activity by activating FoxO

FoxO is the key downstream effector of insulin, and insulin regulates FoxO phosphorylation via Akt/PKB (Kok et al., 2009). Insulin activates phosphatidylinositol 3-kinase (PI3K), which converts 4,5,-phosphatidylinositol (PIP2) to 3,4,5-phosphatidylinositol (PIP3) to recruit Akt to the cell membrane, where phosphoinoside-dependent kinase (PDK) phosphorylates Akt (Czech, 2000). Phosphorylated Akt phosphorylates FoxO to retain FoxO in the cytosol by interacting with 14-3-3 (Saltiel and Kahn, 2001), which suppresses FoxO transcriptional activity (Tzivion et al., 2011). In contrast, PTEN degrades the phosphate from PIP3 to produce PIP2, which cannot attract Akt to the cell membrane; therefore the Akt cannot be phosphorylated (Maehama and Dixon, 1998); therefore, FoxO is not phosphorylated and is translocated to the nucleus to initiate gene transcription to inhibit cell proliferation and promote cell quiescence and apoptosis (Zhang et al., 2011).

Previous studies have shown that insulin suppresses FoxO expression and keeps FoxO in the cytoplasm, and that 20E increases FoxO expression in *H. armigera* (Hou et al., 2012). 20E promotes FoxO nuclear localization in *D. melanogaster* (Colombani et al., 2005; Hossain et al., 2013). This study showed that insulin, via Akt, regulates FoxO phosphorylation and maintains FoxO in the cytoplasm. However, 20E upregulates PTEN expression, which inhibits Akt and FoxO phosphorylation to induce FoxO nuclear translocation. 20E upregulated *PTEN* and *FoxO* expression via EcRB1 and USP1. This study revealed that 20E regulates PTEN and FoxO expression, inhibits FoxO phosphorylation and promotes its nuclear localization to antagonize insulin activity. Thus, PTEN and FoxO play key roles in the crosstalk between the insulin and 20E pathways.

In *Drosophila*, insulin-like peptide 8 (DILP8) coordinates growth and maturation (Colombani et al., 2012). The expression and secretion of *dilp8* delays metamorphosis by suppressing ecdysone production and activity (Garelli et al., 2012). Nutrition controls *Drosophila* body size through the interaction of FoxO with USP in the prothoracic gland to mediate ecdysone biosynthesis (Koyama et al., 2014). Our studies suggested that 20E promotes PTEN expression, which represses Akt and FoxO phosphorylation to counteract insulin activity and induce molting-related gene expression. Hence, considering these works together, FoxO is involved both in ecdysone synthesis in the "brain" and in the ecdysone response in the epidermis. FoxO promotes apoptosis in rat sympathetic neurons (Gilley et al., 2003); therefore, larval molting behavior, i.e. shedding the old cuticle, may depend on the PG producing 20E and thereafter regulating gene expression, not only in epidermal cells, but also in nerve and muscle cells, which will be interesting work for a future study.

FoxO regulates BrZ7 transcription in the 20E signal pathway

The FoxO protein can bind to at least 700 gene promoters to regulate gene transcription (Alic et al., 2011). The consensus FoxO binding sequences are (5′-T**TGTTT**AC-3′) (Eijkelenboom and Burgering, 2013) and (5′- (T/C)(G/A)**AAACA**A)-3′) (Barthel et al., 2005). BrZ7 expression was increased during molting and metamorphosis by 20E regulation (Cai et al., 2014b). The present study showed that FoxO expression was increased during molting and metamorphosis by 20E induction via EcRB1 and USP1. 20E upregulates *BrZ7* transcription via FoxO. ChIP and EMSA assays confirmed that FoxO binds directly to a FoxOBE (-845 ~ -838 before ATG of *BrZ7*) in the 5′ upstream region of *BrZ7* under 20E induction. Our research reveals a new mechanism of 20E regulating BrZ7 expression.

In the 5′ upstream region (-933 bp before ATG) of *H. armigera BrZ7*, no EcRE was predicted by searching using the conserved region (HHR3 EcRE, GGGGTCAATGAACTG) (Liu et al., 2014). Br expression was induced by 20E in *M. sexta* (Zhou et al., 1998). Br is regulated by 20E via a −4950 bp and −3480 bp upstream distal EcRE promoter in *B. mori* (Nishita, 2014). *Br* transcription is also upregulated by the juvenile hormone (JH) to play roles in the JH pathway in *M. sexta*, *D. melanogaster* (Zhou and Riddiford, 2002), *Tribolium castaneum* (Konopova and Jindra, 2008), and *H. armigera* (Cai et al., 2014b). However, a typical E-box (CACGTG) in the JH response element was not detected in the 5′ upstream region (−933 bp before ATG) of *H. armigera BrZ7*. In addition to transcriptional regulation, BrZ7 is also regulated post-translationally. BrZ7 is phosphorylated by JH induction but is not phosphorylated by 20E induction (Cai et al., 2014b). The BrZ7 protein level and transcription activity is maintained by heat shock protein 90 (Cai et al., 2014a). These studies suggested that Br expression is regulated by JH and 20E.

FoxO via BrZ7 mediates CPA expression for insect molting

Proteases have a key role in molting by promoting proteolysis during apolysis (Samuels and Paterson, 1995). CPA participates in apolysis in the molting fluid and its transcript levels are increased at the fifth instar molting stage and the sixth instar prepupal stage by 20E induction in *H. armigera* (Sui et al., 2009). The present study showed that *FoxO* knockdown led to molting failure because of the suppression of *BrZ7* and *CPA* expression. *BrZ7* knockdown did not repress *FoxO* expression but did repress *CPA* expression, which indicated that FoxO regulates CPA expression via BrZ7. This finding revealed that FoxO participates in 20E-induced proteolysis during molting by regulating *BrZ7* and *CPA* expression. CPA is also highly expressed during metamorphosis in *H. armigera* (Sui et al., 2009); therefore, FoxO could also regulate metamorphic molting. However, we did not observe the effect of *FoxO* knockdown on metamorphic molting because the larvae died before metamorphosis after *FoxO* knockdown in fifth instar larvae. *FoxO* was not successfully knocked down in sixth instar larvae; however, the reason for this phenomenon is unclear.

FoxO has various functions in regulating gene expression in vertebrates and insects; the most important of which is to promote apoptosis, inhibit cell cycle and resist oxidative stress (Eijkelenboom and Burgering, 2013). As a tumor suppressor, FoxO regulates a series of gene transcriptions in humans (Huang and Tindall, 2011). FoxO mediates the reduction in cell number associated with reduced insulin signaling *in Drosophila*. Homozygous of *FoxO* null alleles in *Drosophila* are viable, with normal size; however, they are sensitive to oxidative stress (Junger et al., 2003). The knockdown of *FoxO* in *H. armigera* larvae resulted in the larvae failing to molt and shed their old cuticle, and they died before entering the next stage. The varying results obtained from *Drosophila* and *Helicoverpa* might be attributed to the different orders of the insects and the depletion of FoxO at different developmental stages.

CONCLUSIONS

Insulin induces Akt phosphorylation; in turn Akt induces FoxO phosphorylation and cytoplasmic localization, which allows PG growth to produce more 20E. 20E then upregulates PTEN and FoxO expression via EcRB1 and USP1. PTEN represses Akt phosphorylation, which represses FoxO phosphorylation. The non-phosphorylated FoxO is translocated into the nucleus and binds directly to FoxOBE in the upstream region of *BrZ7* to induce *BrZ7* transcription. BrZ7 then regulates CPA expression for proteolysis during molting (Fig. 10).

Materials and methods

Animals

Helicoverpa armigera larvae were raised in the laboratory at 26 ± 1°C under a 14 h/10 h light/dark cycle and fed with an artificial diet, using previously described methods (Zhao et al., 1998).

Recombinant expression of FoxO and preparation of the antiserum

A fragment (amino acids 82-921) of FoxO was expressed in *Escherichia coli* BL21 (DE3) from the pET30a (+) vector. The recombinant FoxO proteins were purified through 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified recombinant FoxO protein was used to prepare rabbit polyclonal antiserum according to the previous method (Cai et al., 2014b). The detail was described in the Supplementary files.

Western blot

Proteins from different tissues were extracted in Tris-buffered saline (TBS: 10 mM Tris–HCl, 150 mM NaCl, pH 7.5) with 1 mM phenylmethanesulfonyl fluoride. The protein concentration was determined using the Bradford method. Equal amounts of proteins (50 μg) were subjected to 7.5% or 12.5% SDS-PAGE and blotted onto a nitrocellulose membrane. The proteins were detected by protein specific polyclonal antibodies (1:200 in blocking solution) and alkaline phosphatase-conjugated goat-anti-rabbit IgG (1:10,000 in the blocking solution). The detail was described in the Supplementary files.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis

The first-strand cDNA was synthesized with M-MLV reverse transcriptase (BioTeke Corporation, Beijing, China) as the qRT-PCR template. qRT-PCR was performed using 2 × SYBR RT-PCR pre-mixture (BioTeke Corporation, Beijing, China) with a CFX96^{TM} real-time system (Bio-Rad Laboratories, Hercules, California, USA). The relative expression levels of the genes were quantified using *H. armigera* β-actin as an internal control. The experiments were repeated three times. Table S1 shows the qRT-PCR primers used in this study. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Liu et al., 2013).

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Hormonal regulation

The epidermal cell line (HaEpi) was established from the 5th instar larval integument of *H. armigera* in our library laboratory (Shao et al., 2008). The cells were frozen from the 5th passage primarily in Grace's medium (20% FBS) and 10% dimethyl sulfoxide (DMSO), which were then cultured and maintained for experiments. The culture method was described in an earlier work (Liu et al., 2011). At densities ranging from 70% to 90%, the cells were treated with 20E or insulin. The controls were treated with the same amount of dimethyl sulfoxide (DMSO). Sixth instar 6 h larvae were injected with 20E or JH III (500 ng/larva). Control larvae were injected with DMSO. Total RNA from the larval epidermis was extracted using Unizol (CoWin Biotech, Beijing, China), and then used for qRT-PCR analysis.

RNA interference (RNAi) of FoxO in larvae

The DNA template was obtained from the primers of gene using primers RNAi-F/R and GFP-RNAi-F/R. dsRNA was synthesized using the MEGAscript™ RNAi kit (Ambion®, Austin, TX, USA) according to the manufacturer's instructions. The PCR primers are listed in Table S1. One µg of FoxO dsRNA (*dsFoxO*) was injected into fifth instar 12 h larvae. Controls were treated with the same volume of green fluorescent protein (GFP) dsRNA (*dsGFP*). Total RNA was extracted to detect the effects of RNAi 2 d after injection. Thirty larvae were injected for each treatment and three independent replicates were performed.

Immunohistochemistry

The epidermis of the larvae was dissected and fixed in 4% paraformaldehyde overnight at 4 °C. The tissues were dehydrated with an ethanol gradient and embedded into paraffin. The paraffin sections were cut into 7-µm-thick slices, placed on gelatin-coated slides, and dried overnight at 42°C. The slides were treated following previously described methods (Zhao et al., 2005). The slides were digested with proteinase K (50 µg/mL) for 30 min at room temperature and incubated overnight at 4°C with specific antibodies [1:200 in 2% bovine serum albumin (BSA)] after being blocked for 30 min in 2% BSA at 37°C. The slides were treated with the secondary antibody goat anti-rabbit-Alexa Fluor 488 or 568 (1:1000 in 2% BSA) in the dark for 2 h at room temperature and stained with 4-6-diamidino-2-phenylindole dihydrochloride [DAPI; AnaSpec Inc., San Jose, CA, USA]. The detail was described in the Supplementary files.

Immunocytochemistry

HaEpi cells were seeded at a density of 2×10^5 in 500 µL Grace's medium supplemented with 10% fetal bovine serum (FBS) at 27°C for 24 h. The cells were incubated with 1 µM 20E or JH III for 1 h to 6 h. In the other group, the cells were incubated in Grace's medium (without FBS) for 24 h to induce starvation and in 2.5 µg/mL insulin for 6 h, followed by treatment with 1 µM 20E for another 6 h. The cells were fixed in 4% paraformaldehyde for 15 min and washed three times with 1 × PBS. The cells were permeabilized in 1 × PBS with 0.2% Triton X-100 for 10 min and blocked with 2% BSA in 1 × PBS for 30 min. The anti-FoxO antibody (1:200 dilution) was added into the cells overnight at 4°C. The cells were incubated in goat anti-rabbit IgG–Alexa Fluor 488 for 1 h at 37°C after being washed six times for 5 min each. The nuclei were stained with DAPI for 10 min at room temperature. The negative control was treated following the same method, but the primary antibody was replaced with preserum.

RNAi in HaEpi cell line

Transient transfection was performed using RNAfectin transfection reagent (Tiangen, Beijing, China) according to the manufacturer's instructions. The HaEpi cells were cultured in 1 mL Grace's medium with dsRNA and RNAfectin transfection reagent without FBS at 80% density. The final concentrations of the dsRNA and the RNAfectin transfection reagent were 2 µg/mL and 4 µg/mL, respectively. After 12 h, the cells were replenished with a fresh medium with FBS that contained 1 µM 20E. The control group was treated with equivalent amounts of DMSO. After 6 h of growth, RNA was isolated and subjected to qRT-PCR analysis. Three replicates were performed independently.

Protein overexpresssion and chromatin immunoprecipitation (ChIP)

The ORF of FoxO was inserted into vector pIEx-4-GFP-His to overexpress FoxO (with C-terminal GFP and histidine tags). The cells were transfected with different vectors, and then incubated in 1 µM 20E or JH III for 3 h. Protein-DNA complexes were immunoprecipitated by anti-FoxO antibodies. DNA was purified using phenol/chloroform and ethanol precipitation as templates for qRT-PCR. The 5′ upstream region of *BrZ7* was cloned using the genome walking method. The BrZ7PF/PR and BrZ7F/R primers are listed in Table S1. The input was the amount of chromatin DNA before immunoprecipitation. The data was calculated according the followed formula: % of chromatin input = $100 \times 2^{-(\mathrm{Ct\ [ChIP]} - (\mathrm{Ct\ [Input]} - \mathrm{Log}_2(\mathrm{Input\ Dilution\ Factor})))}$. $\mathrm{Ct}_{\mathrm{ChIP}}$: the Ct of qRT-PCR from anti-body precipitate, $\mathrm{Ct}_{\mathrm{Input}}$: the Ct of qRT-PCR before immunoprecipitation. Input Dilution Factor = (fraction of the input chromatin saved)$^{-1}$. The detail was described in the Supplementary files.

Electrophoretic mobility shift assay (EMSA)

HaEpi cells were transfected with pIEx-4-FoxO-GFP-His plasmids. After 48 h, the cells were treated with 1 µM 20E or JH III; the control received the same volume of DMSO. After 6 h, the cells were lysed with lysis Buffer (50 mM KCl, 0.5% NP-40, 25 mM HEPES pH 7.8, 10 µg/mL Leupeptin, 20 µg/mL Aprotinin, 125 µM DTT, 1 mM PMSF). The nuclear proteins were isolated and FoxO-GFP-His protein was purified for FoxOBE binding experiments. The reaction was applied to a 6.5% polyacrylamide gel. The samples were then transferred into a nylon membrane to detect the DNA band by phosphatase-labeled anti-Dig antibody. The detail was described in the Supplementary files.

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Competing interests

No competing interests declared.

Author contributions

MJC performed and analyzed the experiments shown in Figures 4, 5, 8 and 9. WLZ performed and analyzed the experiments shown in Figures 5 and 7. YPJ performed and analyzed the experiments shown in Figures 6 and 8. QS performed and analyzed the experiments shown in Figures 1, 2, 3 and 4. XQZ performed and analyzed the experiments in Figure 7. JXW and XFZ conceived and coordinated the study and edited the paper, and drafted Figure 10. All authors reviewed the results and approved the final version of the manuscript. ALL co-authors have checked and confirmed their contribution statement.

REFERENCES

Ali, I. U., Schriml, L. M. and Dean, M. (1999). Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. *J Natl Cancer Inst* **91**, 1922-1932.

Alic, N., Andrews, T. D., Giannakou, M. E., Papatheodorou, I., Slack, C., Hoddinott, M. P., Cocheme, H. M., Schuster, E. F., Thornton, J. M. and Partridge, L. (2011). Genome-wide dFOXO targets and topology of the transcriptomic response to stress and insulin signalling. *Mol Syst Biol* **7**, 502.

Barthel, A., Schmoll, D. and Unterman, T. G. (2005). FoxO proteins in insulin action and metabolism. *Trends in endocrinology and metabolism: TEM* **16**, 183-189.

Cai, M. J., Li, X. R., Pei, X. Y., Liu, W., Wang, J. X. and Zhao, X. F. (2014a). Heat shock protein 90 maintains the stability and function of transcription factor Broad Z7 by interacting with its Broad-Complex-Tramtrack-Bric-a-brac domain. *Insect Mol Biol* **23**, 720-732.

Cai, M. J., Liu, W., Pei, X. Y., Li, X. R., He, H. J., Wang, J. X. and Zhao, X. F. (2014b). Juvenile hormone prevents 20-hydroxyecdysone-induced metamorphosis by regulating the phosphorylation of a newly identified broad protein. *The Journal of biological chemistry* **289**, 26630-26641.

Colombani, J., Andersen, D. S. and Leopold, P. (2012). Secreted peptide Dilp8 coordinates *Drosophila* tissue growth with developmental timing. *Science* **336**, 582-585.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carre, C., Noselli, S. and Leopold, P. (2005). Antagonistic actions of ecdysone and insulins determine final size in *Drosophila*. *Science* **310**, 667-670.

Czech, M. P. (2000). PIP2 and PIP3: complex roles at the cell surface. *Cell* **100**, 603-606.

Dubrovsky, E. B. (2005). Hormonal cross talk in insect development. *Trends Endocrinol Metab* **16**, 6-11.

Eijkelenboom, A. and Burgering, B. M. (2013). FOXOs: signalling integrators for homeostasis maintenance. *Nat Rev Mol Cell Biol* **14**, 83-97.

Erezyilmaz, D. F., Riddiford, L. M. and Truman, J. W. (2006). The pupal specifier broad directs progressive morphogenesis in a direct-developing insect. *P Natl Acad Sci USA* **103**, 6925-6930.

Fahrbach, S. E., Smagghe, G. and Velarde, R. A. (2012). Insect Nuclear Receptors. *Annual Review of Entomology, Vol 57* **57**, 83-106.

Furuyama, T., Yamashita, H., Kitayama, K., Higami, Y., Shimokawa, I. and Mori, N. (2002). Effects of aging and caloric restriction on the gene expression of Foxo1, 3, and 4 (FKHR, FKHRL1, and AFX) in the rat skeletal muscles. *Microsc Res Tech* **59**, 331-334.

Gao, X., Neufeld, T. P. and Pan, D. (2000). *Drosophila* PTEN regulates cell growth and proliferation through PI3K-dependent and-independent pathways. *Developmental biology* **221**, 404-418.

Garelli, A., Gontijo, A. M., Miguela, V., Caparros, E. and Dominguez, M. (2012). Imaginal discs secrete insulin-like peptide 8 to mediate plasticity of growth and maturation. *Science* **336**, 579-582.

Gilley, J., Coffer, P. J. and Ham, J. (2003). FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* **162**, 613-622.

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 Molec* **43**, 829-838.

Hou, L., Cai, M. J., Liu, W., Song, Q. and Zhao, X. F. (2012). Small GTPase Rab4b participates in the gene transcription of 20-hydroxyecdysone and insulin pathways to regulate glycogen level and metamorphosis. *Dev Biol* **371**, 13-22.

Huang, H. and Tindall, D. J. (2011). Regulation of FOXO protein stability via ubiquitination and proteasome degradation. *Biochim Biophys Acta* **1813**, 1961-1964.

Junger, M. A., Rintelen, F., Stocker, H., Wasserman, J. D., Vegh, M., Radimerski, T., Greenberg, M. E. and Hafen, E. (2003). The *Drosophila* forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *J Biol* **2**, 20.

Kaufmann, E. and Knochel, W. (1996). Five years on the wings of fork head. *Mech Dev* **57**, 3-20.

Kok, K., Geering, B. and Vanhaesebroeck, B. (2009). Regulation of phosphoinositide 3-kinase expression in health and disease. *Trends Biochem Sci* **34**, 115-127.

Konopova, B. and Jindra, M. (2008). Broad-complex acts downstream of Met in juvenile hormone signaling to coordinate primitive Holometabolan metamorphosis. *Development* **135**, 559-568.

Koyama, T., Rodrigues, M. A., Athanasiadis, A., Shingleton, A. W. and Mirth, C. K. (2014). Nutritional control of body size through FoxO-Ultraspiracle mediated ecdysone biosynthesis. *Elife* **3**.

Liu, C. Y., Liu, W., Zhao, W. L., Wang, J. X. and Zhao, X. F. (2013). Upregulation of the expression of prodeath serine/threonine protein kinase for programmed cell death by steroid hormone 20-hydroxyecdysone. *Apoptosis* **18**, 171-187.

Liu, C. Y., Zhao, W. L., Wang, J. X. and Zhao, X. F. (2015). Cyclin-dependent kinase regulatory subunit 1 promotes cell proliferation by insulin regulation. *Cell Cycle*, 1-13.

Liu, P. C., Wang, J. X., Song, Q. S. and Zhao, X. F. (2011). The participation of calponin in the cross talk between 20-hydroxyecdysone and juvenile hormone signaling pathways by phosphorylation variation. *PLoS One* **6**.

Liu, W., Cai, M. J., Wang, J. X. and Zhao, X. F. (2014). In a nongenomic action, steroid hormone 20-hydroxyecdysone induces phosphorylation of cyclin-dependent kinase 10 to promote gene transcription. *Endocrinology* **155**, 1738-1750.

Maehama, T. and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *Journal of Biological Chemistry* **273**, 13375-13378.

Mammucari, C., Milan, G., Romanello, V., Masiero, E., Rudolf, R., Del Piccolo, P., Burden, S. J., Di Lisi, R., Sandri, C., Zhao, J., et al. (2007). FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* **6**, 458-471.

Matsuzaki, H., Daitoku, H., Hatta, M., Tanaka, K. and Fukamizu, A. (2003). Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *P Natl Acad Sci USA* **100**, 11285-11290.

Mirth, C., Truman, J. W. and Riddiford, L. M. (2005). The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. *Curr Biol* **15**, 1796-1807.

Mirth, C. K., Tang, H. Y., Makohon-Moore, S. C., Salhadar, S., Gokhale, R. H., Warner, R. D., Koyama, T., Riddiford, L. M. and Shingleton, A. W. (2014). Juvenile hormone regulates body size and perturbs insulin signaling in *Drosophila*. *P Natl Acad Sci USA* **111**, 7018-7023.

Nishita, Y. (2014). Ecdysone response elements in the distal promoter of the Bombyx Broad-Complex gene, BmBR-C. *Insect Mol Biol* **23**, 341-356.

Ote, M., Mita, K., Kawasaki, H., Daimon, T., Kobayashi, M. and Shimada, T. (2005). Identification of molting fluid carboxypeptidase A (MF-CPA) in Bombyx mori. *Comp Biochem Phys B* **141**, 314-322.

Puig, O. and Mattila, J. (2011). Understanding Forkhead box class O function: lessons from *Drosophila melanogaster*. *Antioxid Redox Signal* **14**, 635-647.

Riddiford, L. M., Cherbas, P. and Truman, J. W. (2001). Ecdysone receptors and their biological actions. *Vitam Horm* **60**, 1-73.

Riddiford, L. M., Hiruma, K., Zhou, X., Nelson, C.A., (2003). Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology* **33**, 1327–1338.

Saltiel, A. R. and Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799-806.

Samuels, R. I. and Paterson, I. C. (1995). Cuticle degrading proteases from insect moulting fluid and culture filtrates of entomopathogenic fungi. *Comp Biochem Physiol B Biochem Mol Biol* **110**, 661-669.

Shao, H. L., Zheng, W. W., Liu, P. C., Wang, Q., Wang, J. X. and Zhao, X. F. (2008). Establishment of a new cell line from lepidopteran epidermis and hormonal regulation on the genes. *Plos One* **3**, e3127.

Sui, Y. P., Liu, X. B., Chai, L. Q., Wang, J. X. and Zhao, X. F. (2009). Characterization and influences of classical insect hormones on the expression profiles of a molting carboxypeptidase A from the cotton bollworm (*Helicoverpa armigera*). *Insect Mol Biol* **18**, 353-363.

Tzivion, G., Dobson, M. and Ramakrishnan, G. (2011). FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Bba-Mol Cell Res* **1813**, 1938-1945.

Vilchez, D., Morantte, I., Liu, Z., Douglas, P. M., Merkwirth, C., Rodrigues, A. P. C., Manning, G. and Dillin, A. (2012). RPN-6 determines C. elegans longevity under proteotoxic stress conditions. *Nature* **489**, 263-+.

Zhang, X., Rielland, M., Yalcin, S. and Ghaffari, S. (2011). Regulation and function of FoxO transcription factors in normal and cancer stem cells: what have we learned? *Curr Drug Targets* **12**, 1267-1283.

Zhao, J., Brault, J. J., Schild, A., Cao, P., Sandri, M., Schiaffino, S., Lecker, S. H. and Goldberg, A. L. (2007). FoxO3 coordinately activates protein degradation by the Autophagic/Lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metabolism* **6**, 472-483.

Zhao, X. F., An, X. M., Wang, J. X., Dong, D. J., Du, X. J., Sueda, S. and Kondo, H. (2005). Expression of the *Helicoverpa* cathepsin b-like proteinase during embryonic development. *Arch Insect Biochem Physiol* **58**, 39-46.

Zhao, X. F., Wang, J. X. and Wang, Y. C. (1998). Purification and characterization of a cysteine proteinase from eggs of the cotton boll worm, *Helicoverpa armigera*. *Insect Biochem Mol Biol* **28**, 259-264.

Zhou, B., Hiruma, K., Shinoda, T. and Riddiford, L. M. (1998). Juvenile hormone prevents ecdysteroid-induced expression of broad complex RNAs in the epidermis of the tobacco hornworm, Manduca sexta. *Dev Biol* **203**, 233-244.

Zhou, X. and Riddiford, L. M. (2002). Broad specifies pupal development and mediates the ‘status quo’action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. *Development* **129**, 2259-2269.

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Figures

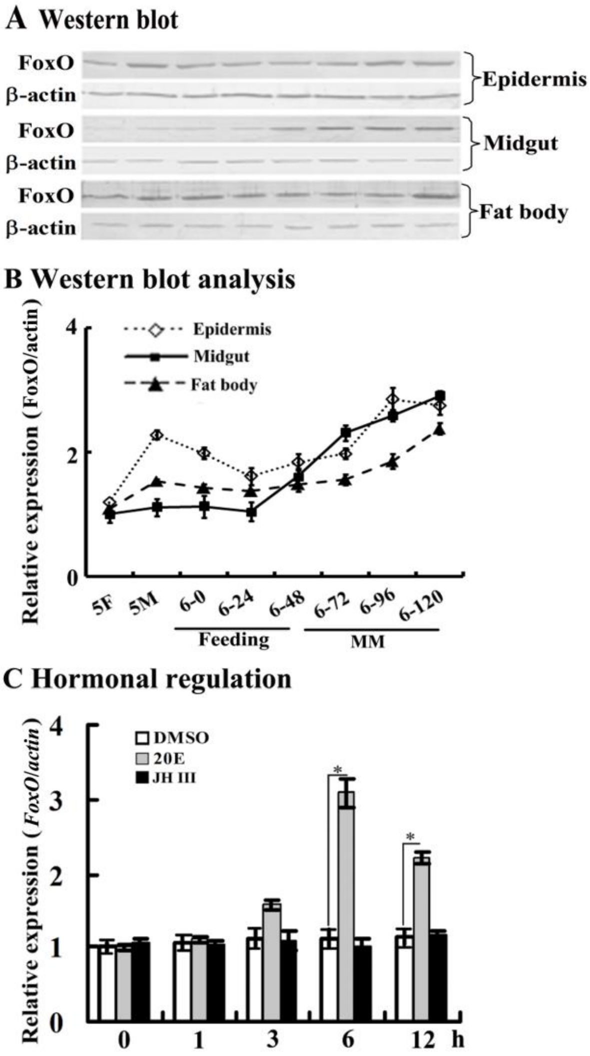

Figure 1. FoxO expression increased in tissues during molting and metamorphosis under 20E regulation. A. Western blot analysis using an antibody against *H. armigera* FoxO. β-actin was used as the control (12.5% gel). **B.** The quantification of the data in A to show the trend of FoxO expression profile. **5F:** Fifth instar feeding stage; **5M:** Fifth instar molting stage; **6-0 h** to **6-120 h:** Sixth instar larvae at various development times. **MM:** Metamorphic molting from the final instar larvae to pupae. **C.** qRT-PCR showing that 20E-induced FoxO expression. Sixth instar 6 h larvae were injected with 20E or JH III (500 ng/larva) for 0, 1, 3, 6, and 12 h. An asterisk indicates significant differences ($P < 0.05$), as assessed by Student's *t*-test based on three independent experiments.

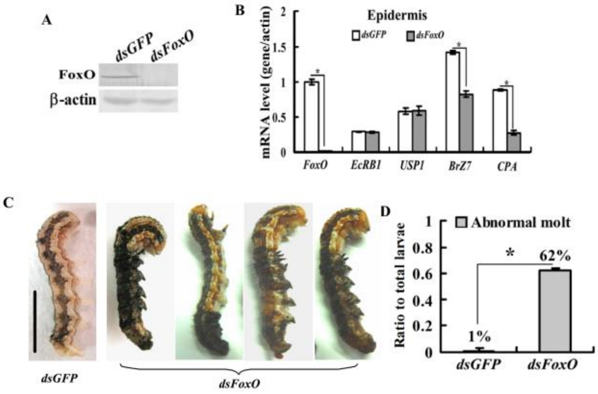

Figure 2. ***FoxO* knockdown blocked gene expression in the 20E pathway and fifth instar to sixth instar molting. A.** Western blot analysis that showing the efficacy of *FoxO* knockdown using the proteins from the epidermis 2 d post dsRNA injection. **B.** qRT-PCR detection of gene expression levels in the epidermis after knockdown of *FoxO* in larvae. An asterisk indicates significant differences ($P < 0.05$), as assessed by Student's *t*-test based on three independent experiments. **C.** Phenotype of *FoxO* knockdown cells. One μg of *dsFoxO* was injected into fifth instar larvae. The control larvae received the same amount of *dsGFP*. Scale bar = 1 cm. **D.** Statistical analysis of molting failure (abnormal molt) ($n = 30 \times 3$).

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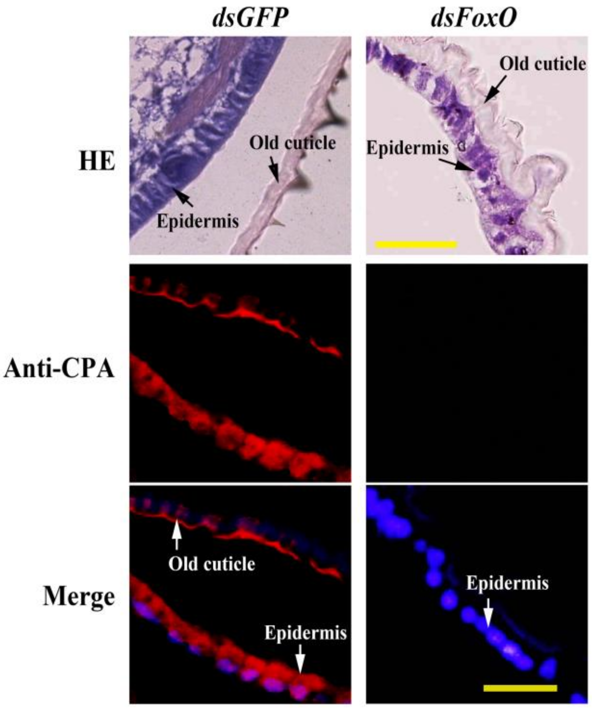

Figure 3. ***FoxO* knockdown blocked apolysis by repressing CPA expression, as assessed by immunohistochemical analysis.** HE staining showing the blocking of apolysis in fifth instar molting larvae after *FoxO* knockdown. The control larvae were treated with the same amount of *dsGFP*. Red fluorescence indicates CPA expression after *FoxO* knockdown using an anti-*Helicoverpa* CPA antibody and a goat anti-rabbit–Alexa Fluor 568 secondary antibody. The nuclei were stained with DAPI. Scale bar = 20 μm.

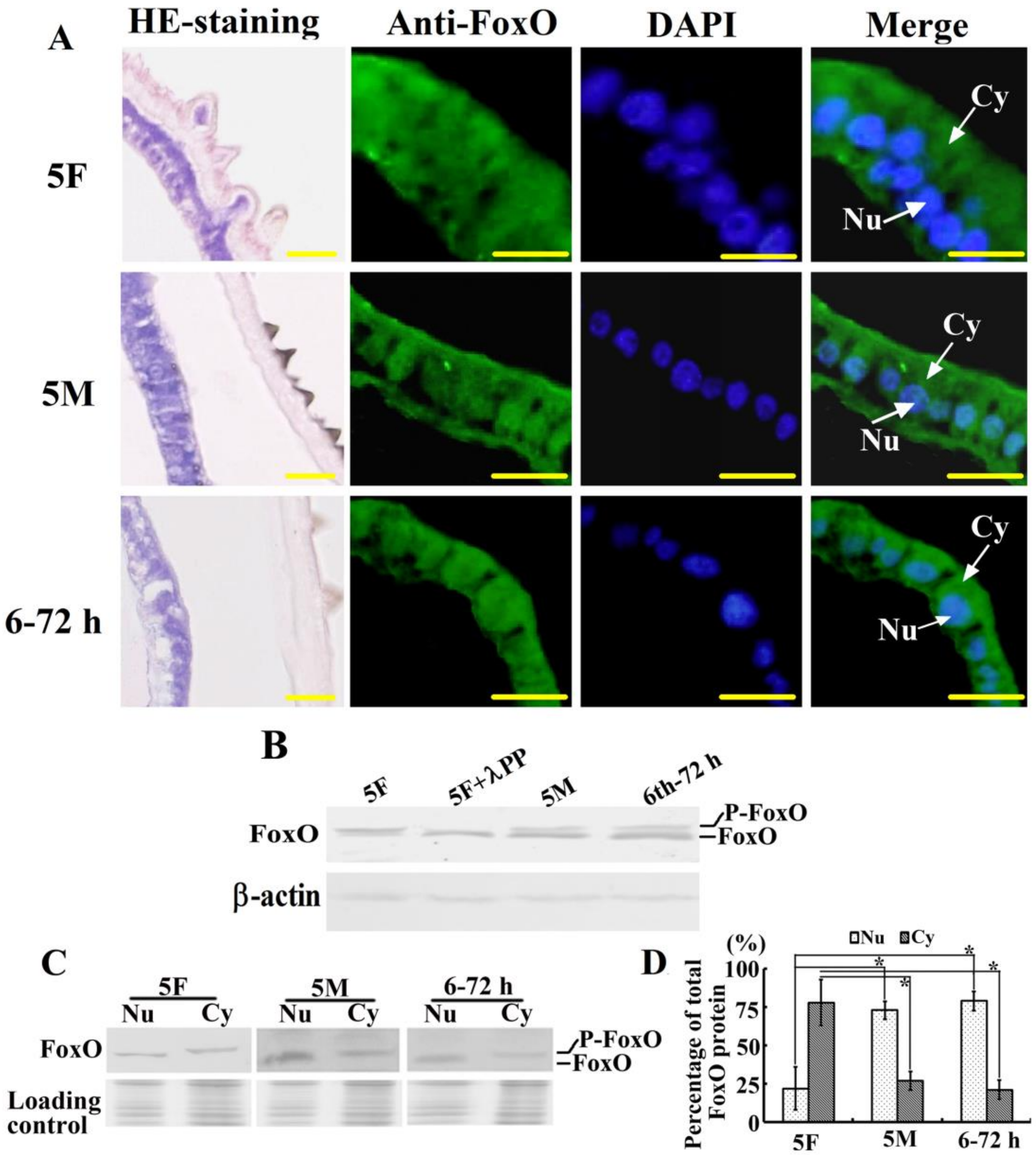

Figure 4. FoxO was not phosphorylated and was distributed partially in the nucleus during molting and metamorphosis. A. The subcellular location of FoxO. HE: Hematoxylin-eosin staining. Green fluorescence indicates FoxO stained using an anti-FoxO antibody and goat anti-rabbit–Alexa Fluor 488 secondary antibody. Blue fluorescence indicates the nucleus stained by DAPI. **B.** Western blot analysis shows the variation of the phosphorylation status of FoxO. Proteins from the epidermis at 5F, 5M, and 6th-72 h stages were extracted for western blot analysis using 7.5% SDS-PAGE gels. Proteins from 5F were isolated and incubated with λPP at 5 μM for 30 min. **C.** Western blot analysis shows the subcellular distribution of the phosphorylated FoxO and the non-phosphorylated FoxO in the epidermis at 5F, 5M, and 6th-72 h larvae. **D.** Statistical analysis of the data in C.

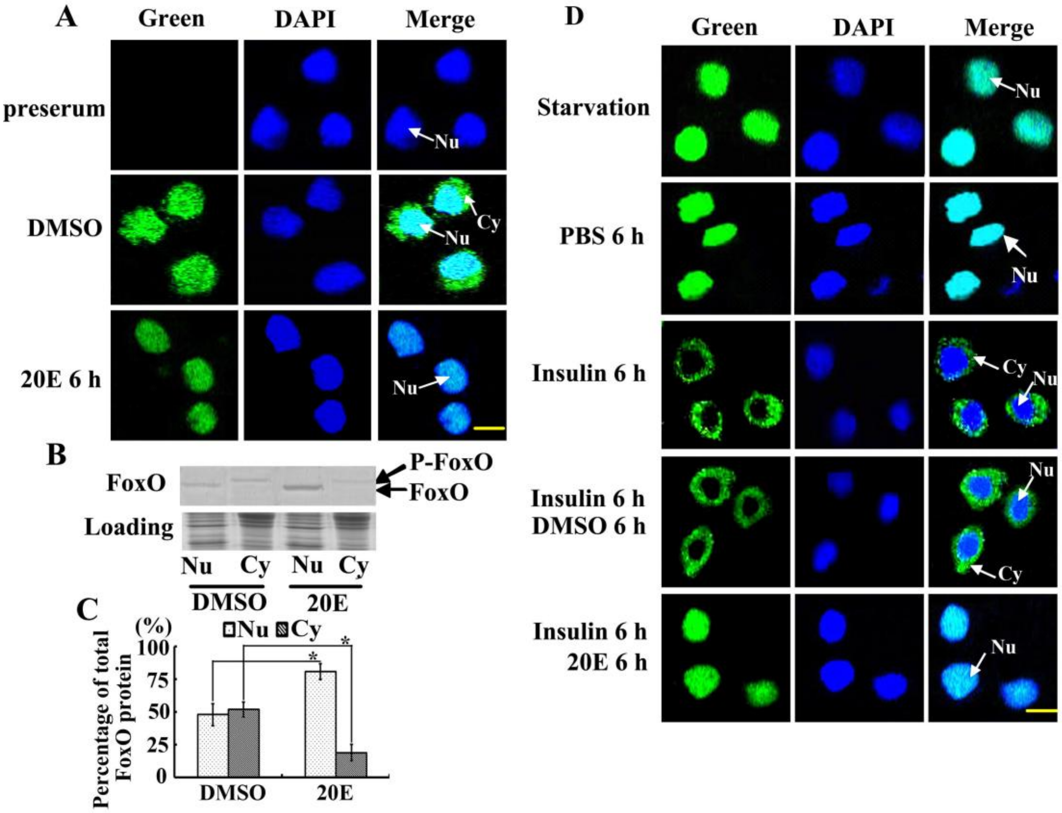

Figure 5. 20E regulated FoxO nuclear localization and inhibited FoxO phosphorylation in HaEpi cells. A. FoxO translocated to the nucleus after 20E induction in Grace’s medium with 10% FBS. Cells were incubated with 1 μM 20E for 6 h. Green fluorescence indicates FoxO stained with an anti-FoxO antibody and the goat anti-rabbit-Alexa Fluor 488 secondary antibody. Blue fluorescence indicates the nucleus stained with DAPI. **Nu:** nuclear fraction; **Cy:** cytoplasmic fraction. **B.** 20E inhibited FoxO phosphorylation according to western blotting analysis with 7.5% SDS PAGE gels. Cells were incubated with 1 μM 20E for 6 h in Grace’s medium with 10% FBS. The control cells received the same amount of DMSO. **C.** The statistical analysis of the data in B. **D.** 20E induced FoxO nuclear localization. The cells were incubated in Grace’s medium without FBS (starvation) for 24 h, with PBS (hormone free control) or insulin (2.5 μg/mL) for 6 h, and with insulin plus DMSO or insulin plus 1 μM 20E for another 6 h in Grace’s medium without FBS. Scale bar = 20 μm.

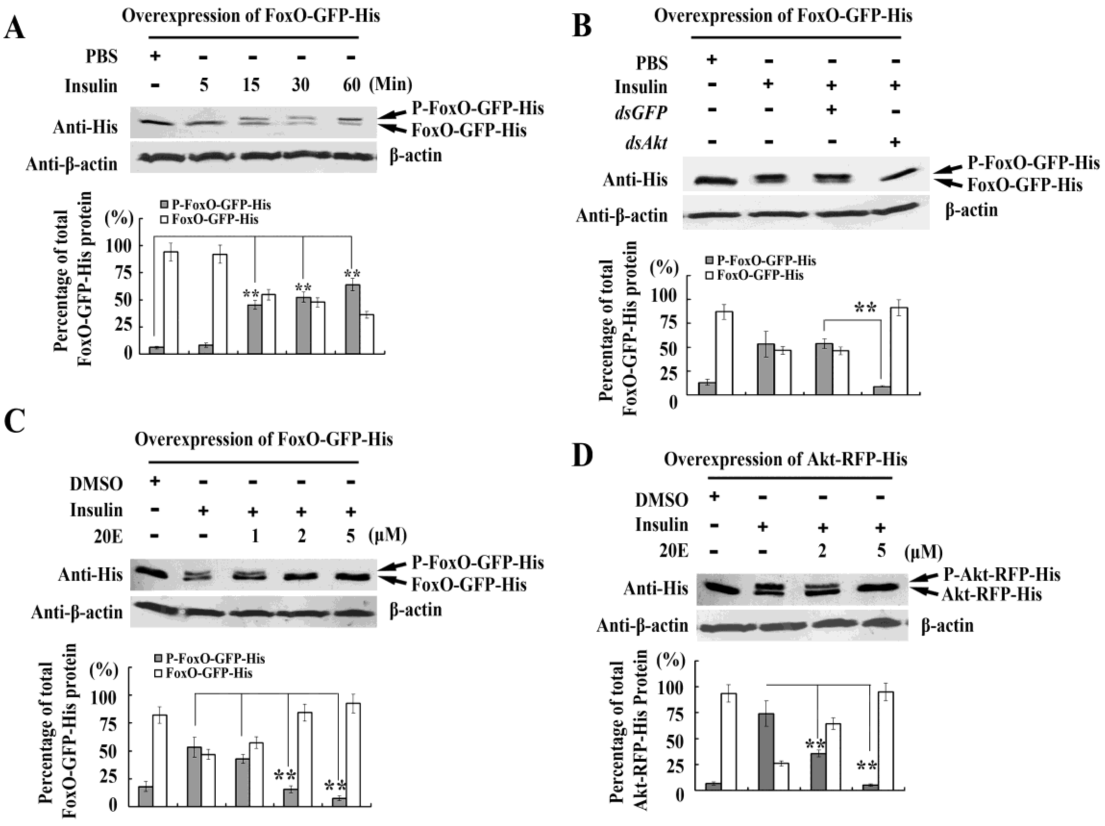

Figure 6. 20E inhibited FoxO phosphorylation via repressing Akt phosphorylation, as analyzed by western blotting. A. Insulin induced FoxO phosphorylation in HaEpi cells. The cells were transfected with pIEx-4-FoxO-GFP-His plasmid for 48 h, and then treated with insulin (2.5 μg/mL) for 5, 15, 30 and 60 min, respectively. **B.** Insulin, via Akt, induced FoxO phosphorylation. The cells were transfected with pIEx-4-FoxO-GFP-His plasmid for 48 h, and then transfected with *dsGFP* or *dsAkt*. The cells were treated with insulin (2.5 μg/mL) for 60 min for western blotting. **C.** 20E inhibited insulin-induced FoxO phosphorylation. The cells were transfected with pIEx-4-FoxO-GFP-His plasmid, and then treated with 1, 2 or 5 μM 20E for 6 h. The cells were then treated with insulin (2.5 μg/mL) for 60 min before western blot analysis. **D.** 20E repressed the insulin-induced Akt phosphorylation. The cells were transfected with pIEx-4-Akt-RFP-His plasmid, and then treated with 2 or 5 μM 20E for 6 h. The cells were treated with insulin (2.5 μg/mL) for 60 min before western blotting. In this figure, the gel concentration for SDS-PAGE was 7.5%.

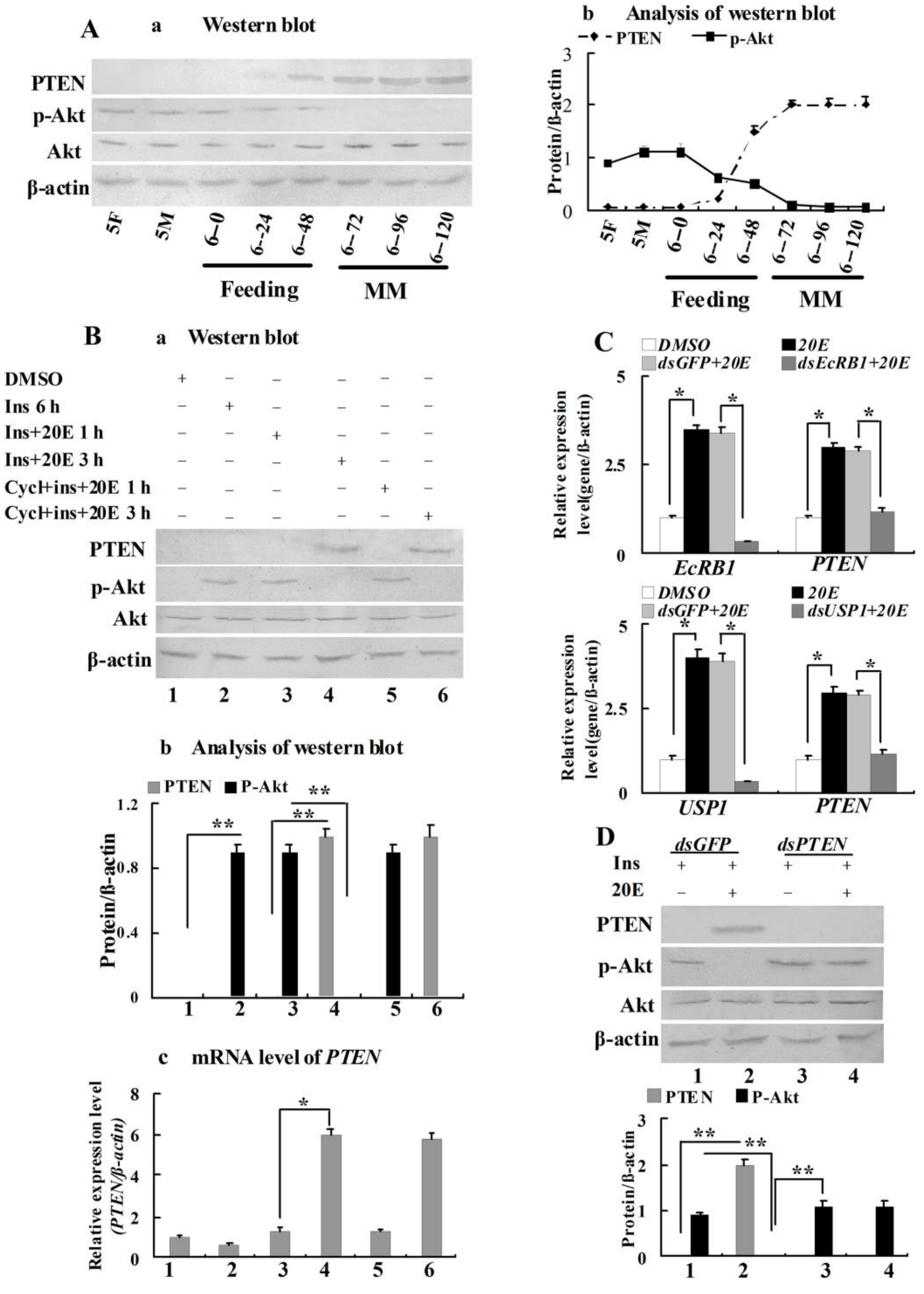

Figure 7. 20E upregulates PTEN expression to repress Akt phosphorylation in HaEpi cells, as assessed by western blotting. A. Expression levels of PTEN and phosphorylated-Akt in the integument during larval development, detected by anti-*Homo*-PTEN, anti-*Homo*-phosphorylated-Akt, anti-*H. armigera*-Akt and anti-*H. armigera*-β-actin antibodies, respectively. The developmental stages are the same as in Figure

1. **a.** Western blot, **b.** Quantification of a**. B.** Hormone induction of the expression of PTEN and Akt phosphorylation. **a.** Lanes indicate various treatments: 1, DMSO as solvent control; 2, insulin (2.5 μg/mL) for 6 h; 3 and 4, insulin (2.5 μg/mL) plus 20E (1 μM) for another 1 or 3 h, respectively; 5 and 6, cycloheximide (5 μg/mL) for 1 h, then insulin (2.5 μg/mL) and 20E (1 μM) for another 1 or 3 h, respectively. **Ins:** insulin; **Cycl:** cycloheximide. **b.** The statistical analysis of PTEN and phosphorylated-Akt (P-Akt) expression by ImageJ software. **c.** qRT-PCR analyses showing the effect of cycloheximide on the mRNA levels of *PTEN* after the same treatments as in a. **C.** 20E, via EcRB1 and USP1, regulates *PTEN* expression, as assessed by qRT-PCR analysis. The cells were treated with *dsEcRB1* or *dsUSP1* (1 μg/mL for 12 h), 20E (1 μM for 6 h). **D.** *PTEN* knockdown kept Akt phosphorylation under 20E treatment. Lanes are: 1, *dsGFP* plus insulin; 2, *dsGFP* plus insulin and 20E; 3, *dsPTEN* plus insulin; 4: *dsPTEN* plus insulin and 20E. The cells were treated with *dsRNA* (1 μg/mL for 12 h), insulin (2.5 μg/mL) alone or plus 20E (1 μM) for other 6 h. The column chart below is the statistical analysis of the western blotting by ImageJ software. Asterisks indicate significant differences between the groups ($p < 0.05$) by Student's *t*-test, based on three independent experiments.

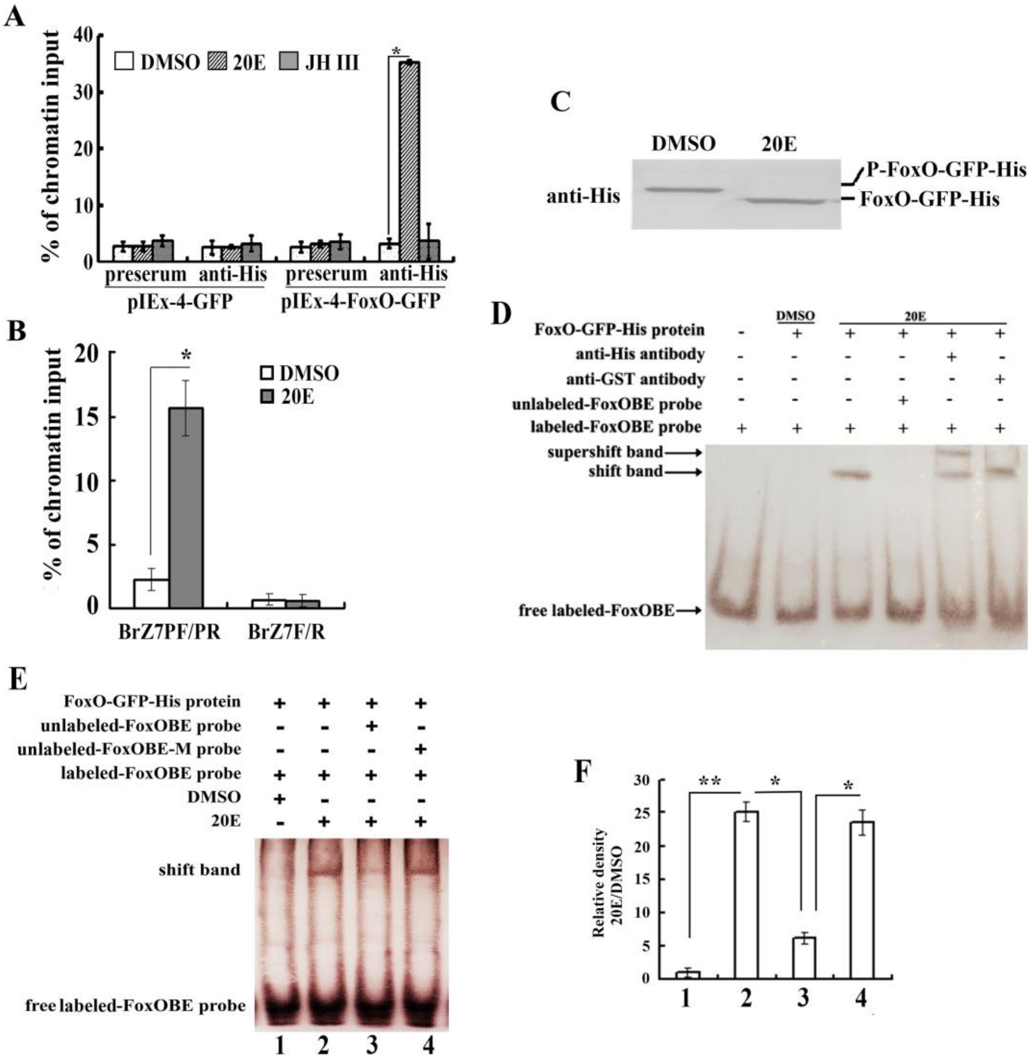

Figure 8. FoxO binds to the FoxOBE in the 5′ upstream region of *BrZ7*. A. ChIP assay of FoxO binding to the 5′ upstream region of *BrZ7*, using primers PF/PR. Cells were transfected with pIEx-4-FoxO-GFP-His or pIEx-4-GFP-His for 48 h, and then treated with DMSO, 1 μM 20E or JH III, respectively. An anti-His antibody was used to immunoprecipitate FoxO-GFP-His, GFP-His and the bound FoxOBE-containing chromatin. Preserum was used as the negative control for nonspecific precipitation. The FoxOBE-containing chromatin in the immunoprecipitate was purified and analyzed by qRT-PCR. **B.** qRT-PCR confirmation that FoxO binds to the 5′ upstream region of *BrZ7*, using primers PF2/PR2 to exclude non-specific FoxO binding to DNA. **C.** Western blot showing the phosphorylation status of FoxO under DMSO or 20E induction in whole cells (7.5% SDS PAGE gel). **D.** EMSAs of FoxO binding to FoxOBE. DMSO and 20E indicate that the FoxO was purified from the nuclei of cells treated with DMSO or 1 μM 20E for 3 h. The shifted band was produced by

FoxO binding to Dig-labeled FoxOBE (5′-TTTTTGCATTTGTTTAAAATAGCAGC-3′), whereas the supershifted band was produced using the anti-His antibody. An anti-GST antibody was used as the negative control for the anti-His antibody. An unlabeled FoxOBE probe was used to compete against the Dig-labeled-FoxOBE probe. **E.** An unlabeled FoxOBE probe and an unlabeled mutated probe were used to compete against the Dig-labeled-FoxOBE probe. **F.** Statistical analyses of the results in C using Quantity One software based on three independent biological experiments. An asterisk indicates significant differences ($P < 0.05$), as assessed by Student's *t*-test.

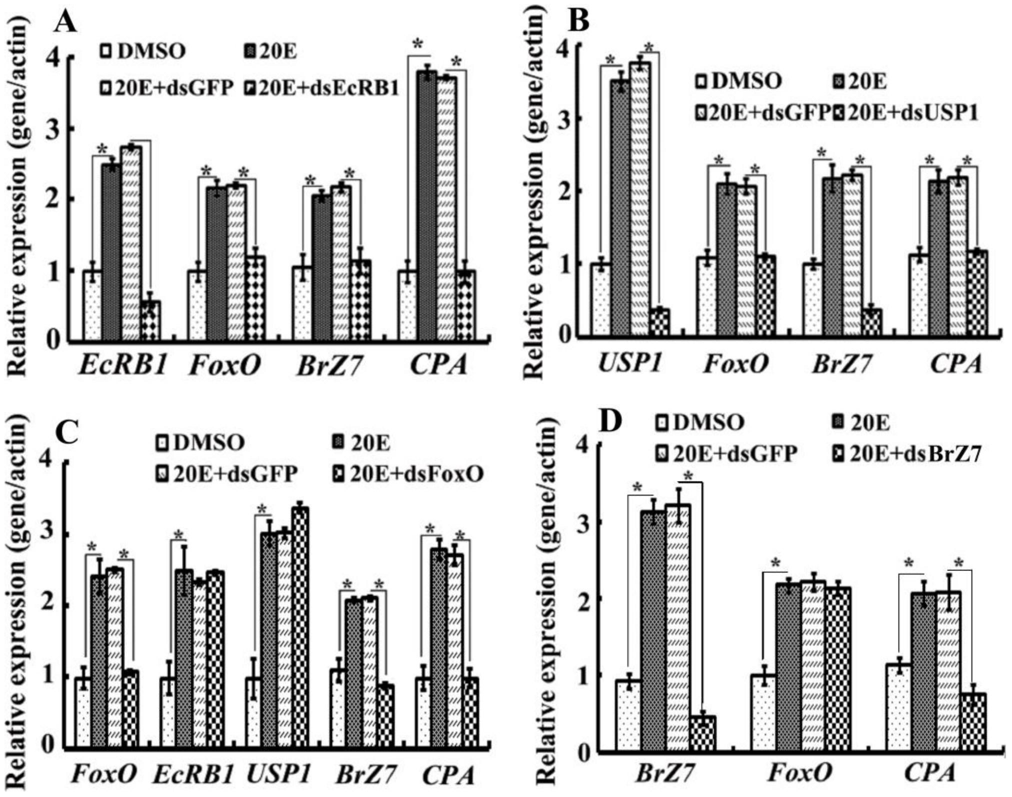

Figure 9. Cascade of gene expressions in 20E regulation via qRT-PCR analysis in HaEpi cells. A and **B.** 20E upregulates *FoxO* expression via EcRB1 and USP1. **C.** 20E regulates *BrZ7* expression via FoxO. **D.** 20E regulates *CPA* expression via BrZ7. The cells were incubated with dsRNA (4 μg/mL) for 10 h and induced by 1 μM 20E for 6 h. The control samples received the same amount of *dsGFP*. Total RNA was isolated for qRT-PCR. An asterisk indicates significant differences ($P < 0.05$), as assessed by Student's *t*-test based on three independent experiments.

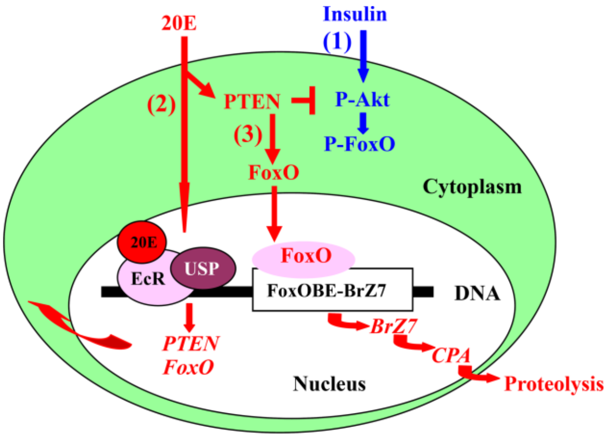

Figure 10. Schematic of 20E activation of FoxO to counteract insulin activity. Insulin induces Akt phosphorylation, which regulates FoxO phosphorylation and cytoplasmic localization (1). 20E, through EcRB1/USP1, upregulates PTEN expression and FoxO expression (2). PTEN represses the phosphorylation of Akt, which in turn represses FoxO phosphorylation, resulting in FoxO nuclear translocation. In the nucleus, FoxO binds to the FoxOBE in the 5′ upstream region of *BrZ7* to directly regulate *BrZ7* transcription. BrZ7 then modulates the expression of CPA for proteolysis during apolysis of insect molting (3).