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RESEARCH ARTICLE

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

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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 inhibits FoxO phosphorylation, resulting in FoxO nuclear translocation. Insulin, via Akt, induces FoxO phosphorylation and cytoplasmic 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 Carboxypeptidase A expression for final proteolysis during insect molting. Hence, 20E activates FoxO via upregulating PTEN expression to counteract insulin activity and promote proteolysis.

KEY WORDS: Forkhead box O, Protein kinase B/Akt, PTEN, Steroid hormone, Proteolysis, Cotton bollworm

INTRODUCTION

Forkhead box O (FoxO) proteins are a subgroup of the Forkhead transcription factor family (Kaufmann and Knöchel, 1996). Mammals have four FoxO genes: *Foxo1*, *Foxo3*, *Foxo4* and *Foxo6* (Furuyama et al., 2002). However, only one FoxO gene (*foxo*) has been identified in *Drosophila* (Jünger 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 the skeletal muscles of mice (Mammucari et al., 2007). FoxO3 stimulates protein degradation in mouse C2C12 myotubes (Zhao et al., 2007). DAF-16, a FoxO protein 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 known as phosphatase and tensin homolog (PTEN) and mutated in multiple advanced cancers 1 (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 the 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 FoxO 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-producing organ, the corpora allata, elevates FoxO activity and results in small pupae (Mirth et al., 2014). Higher concentrations of 20E repress insulin-induced gene expression in *H. armigera* (Liu et al., 2015). In *Drosophila*, 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 remained unclear.

Insect molting serves 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, which is the degradation of the old cuticle by proteases and chitinases in the molting fluid, secreted by the epidermis; and ecdysis, the shedding of the old cuticle (Dubrovsky, 2005). Insect molting is triggered by a higher titer of 20E (Riddiford et al., 2003). 20E binds to its nuclear receptor, ecdysone receptor (EcR), and forms a transcription complex with 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 encodes the transcription factor Broad [Br, or Br-complex (Br-C)], which is crucial 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), which is encoded by 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 remains unclear.

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The present study aimed to determine the mechanism of 20E 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 (ds) RNA 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 upstream region of *BrZ7*. FoxO directly regulates *BrZ7* transcription, which promotes CPA expression for apolysis during molting under 20E induction. Accordingly, FoxO is a crucial regulator in 20E-induced proteolysis during molting.

RESULTS

The conserved Forkhead box of FoxO

We identified *FoxO* from transcriptome sequencing of a cDNA from an epidermal cell line from *H. armigera* (HaEpi cells) (see the supplementary Materials and Methods). The open reading frame (ORF) of *FoxO* comprises 1551 bp (GenBank accession number KM008744). *FoxO* encodes a protein of 516 amino acids with a calculated molecular mass of 55.8 kDa. Three potential Akt phosphorylation sites (Thr49, Ser191 and Ser255) were identified in FoxO. The DNA-binding domain, termed the ‘Forkhead box’, is located near the N-terminal region (amino acids 94-183; Fig. S1). *H. armigera* FoxO shares 85%, 77%, 47% and 36% similarity with *B. mori*, *Danaus plexippus*, *D. melanogaster* and *Homo sapiens* FoxO, respectively (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) and metamorphic stages (6-72 h, 6-96 h and 6-120 h), compared with the feeding stages (5F, 6-24 h and 6-48 h) (Fig. 1A,B). Given that the 20E titer is elevated during molting and metamorphosis in the lepidopteran insect *Manduca sexta* (Riddiford et al., 2003), we suspected that FoxO expression was upregulated by 20E during these stages in *H. armigera*. To validate this hypothesis, we injected 20E into sixth instar 6 h larvae and observed an increase in *FoxO* transcripts; juvenile hormone III (JH III), by contrast, did not induce *FoxO* (Fig. 1C). Therefore, FoxO is likely to be 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 *FoxO* dsRNA (*dsFoxO*) into fifth instar 6 h larvae to knockdown *FoxO* expression. Western blotting and quantitative real-time reverse transcription PCR (qRT-PCR) showed that the expression of FoxO protein and mRNA was successfully knocked down in the epidermis. By contrast, expression of the 20E nuclear receptor *EcRB1* and *USP1* was unaffected by *FoxO* knockdown. However, the transcription factor *BrZ7* and *CPA* were significantly inhibited in expression (Fig. 2A,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. These results indicated that FoxO plays a crucial role in molting by regulating BrZ7 and CPA expression downstream of EcRB1 and USP1 in the 20E pathway.

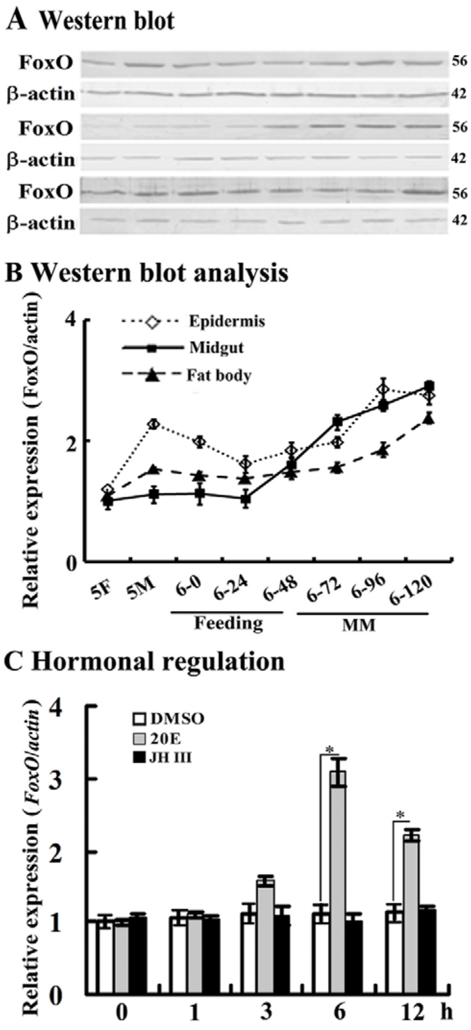

Fig. 1. FoxO expression increases in tissues during molting and metamorphosis under 20E regulation. (A) Western blot analysis (12.5% gel) using an antibody against *H. armigera* FoxO. β-actin was used as the control. Epi, epidermis; Mg, midgut; Fb, fat body. (B) Quantification of the data in A, showing the trend in the 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 times of development; MM, metamorphic molting from the final instar larvae to pupae. (C) qRT-PCR showing that 20E induces *FoxO* expression. Sixth instar 6 h larvae were injected with 20E or JH III (500 ng/larva) and sampled at 0, 1, 3, 6 and 12 h. *$P<0.05$ (Student’s *t*-test), based on three independent experiments. Error bars indicate the mean±s.d. of three independent biological experiments.

When *FoxO* was knocked down, apolysis could not occur and the old cuticle could not be separated from the epidermis, unlike in the control injected with *GFP* dsRNA (*dsGFP*). Moreover, 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 achieve 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

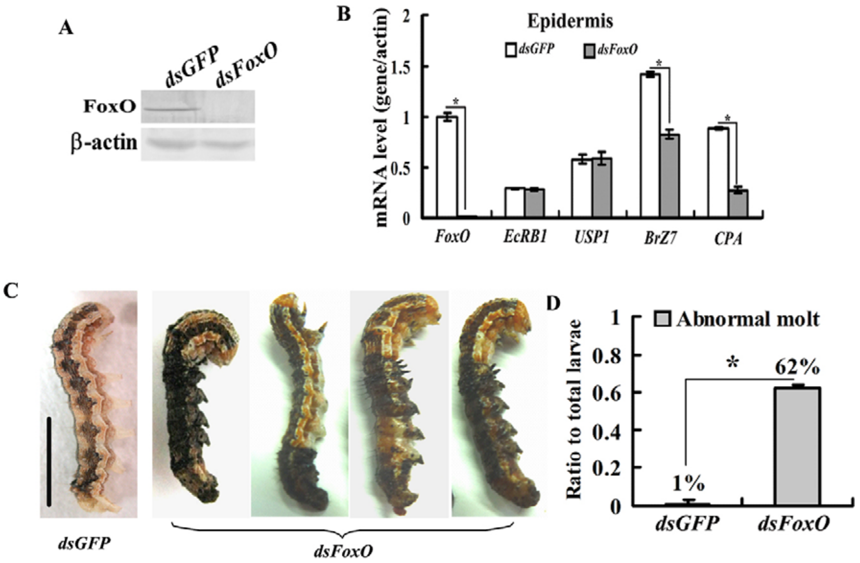

Fig. 2. *FoxO* knockdown blocks gene expression in the 20E pathway and fifth instar to sixth instar molting. (A) Western blot analysis of proteins from the epidermis 2 days post dsRNA injection showing the efficacy of *FoxO* knockdown. (B) qRT-PCR detection of gene expression levels in the epidermis after knockdown of *FoxO* in larvae. *$P<0.05$ (Student's *t*-test), based on three independent experiments. (C) Phenotype of *FoxO* knockdown larvae. 1 µg *dsFoxO* was injected into fifth instar larvae. Control larvae received the same amount of *dsGFP*. Scale bar: 1 cm. (D) Statistical analysis of molting failure (abnormal molt). n=30 in each of three independent experiments. *$P<0.05$ (Student's *t*-test). Error bars indicate the mean±s.d. of three independent biological experiments.

detected both in the cytoplasm and the nucleus in the epidermis, with increased levels in the nucleus during 5M and the metamorphic stages (6-72 h), when the old cuticle was separated from the epidermis, as compared with 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. This suggested that the upper band is the phosphorylated form of FoxO. By contrast, two bands were attributed to FoxO at 5M and 6-72 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,D). Thus, 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.

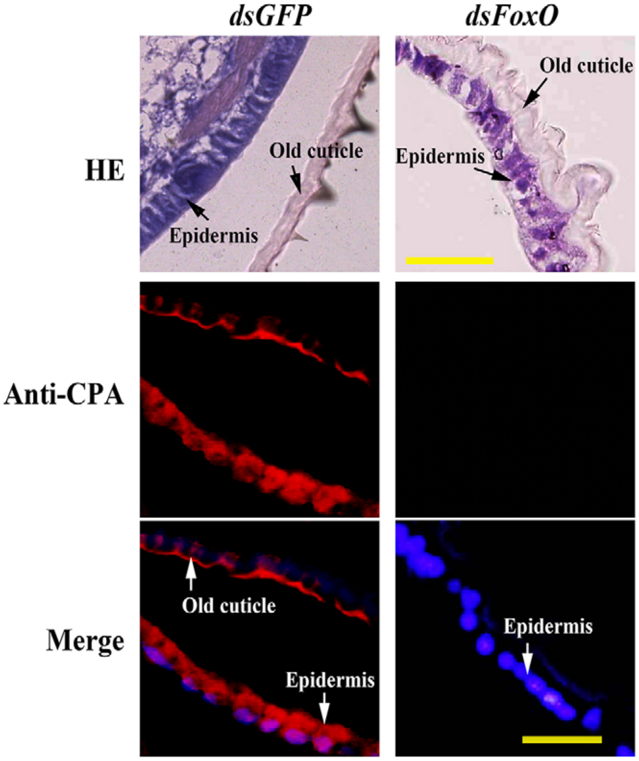

Fig. 3. *FoxO* knockdown blocks apolysis by repressing CPA expression. Hematoxylin and Eosin (HE) staining showing the blocking of apolysis in fifth instar molting larvae after *FoxO* knockdown. Control larvae received the same amount of *dsGFP*. Red fluorescence indicates CPA expression after *FoxO* knockdown (*Helicoverpa* CPA antibody and goat anti-rabbit Alexa Fluor 568 secondary antibody). Nuclei are stained with DAPI (blue). Scale bars: 20 µm.

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 distributed throughout the entire cell, including the cytoplasm and 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,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 inhibition of FoxO phosphorylation by 20E, we analyzed the involvement of Akt in insulin-induced FoxO phosphorylation in HaEpi cells, because insulin, via Akt, regulates the 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,D). These results suggested that 20E represses insulin-

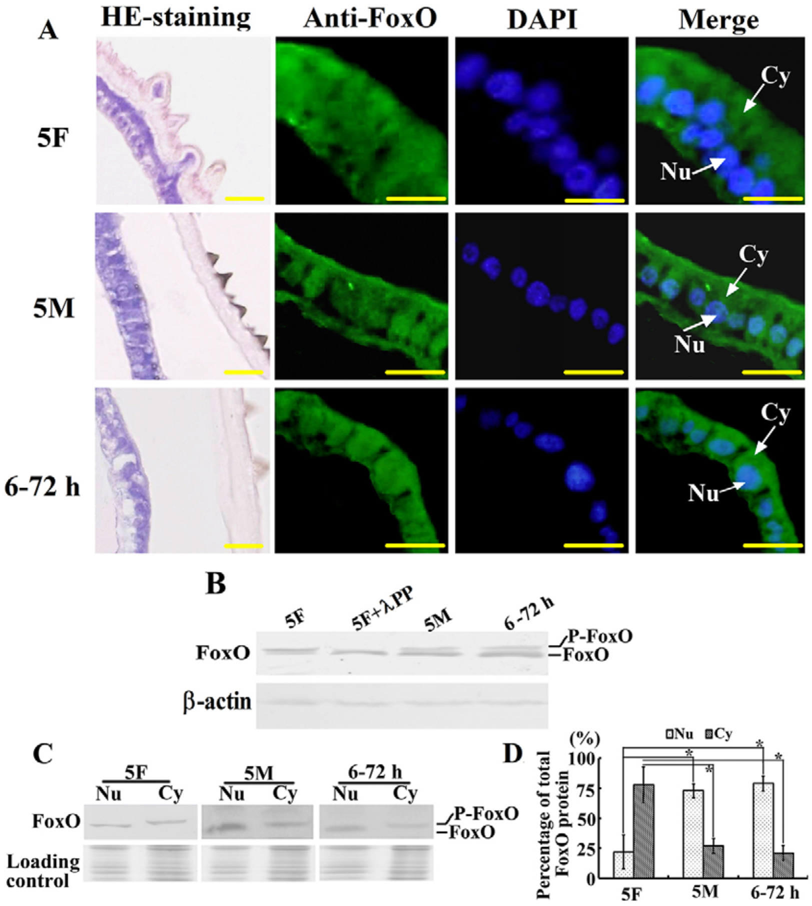

Fig. 4. FoxO is not phosphorylated and is distributed partly in the nucleus during molting and metamorphosis. (A) The subcellular localization of FoxO. Green fluorescence indicates FoxO (anti-FoxO antibody and goat anti-rabbit Alexa Fluor 488 secondary antibody). Nuclei are stained with DAPI (blue). Scale bars: 20 µm. (B) Western blot showing the variation in the phosphorylation status of FoxO. Proteins from the epidermis at 5F, 5M and 6-72 h stages were extracted for western blot analysis via 7.5% SDS-PAGE. Proteins from 5F were isolated and incubated with λPP at 5 µM for 30 min. (C) Western blot analysis showing the subcellular distribution of phosphorylated and non-phosphorylated FoxO in the epidermis of 5F, 5M and 6-72 h larvae. The same proteins were separated and stained with Coomassie Brilliant Blue (CBB) after SDS-PAGE to provide a protein loading control. (D) Statistical analysis of the data in C. $n=3$. $^*P<0.05$ (Student's *t*-test). Error bars indicate mean±s.d. of three independent biological experiments. Cy, cytoplasm or cytoplasmic fraction; Nu, nucleus or nuclear fraction.

induced Akt phosphorylation, which inhibits insulin-induced FoxO phosphorylation.

20E inhibits Akt phosphorylation by 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 direct regulation by 20E of PTEN expression (Fig. 7B). Knockdown of *EcRB1* or *USP1* significantly decreased 20E-induced *PTEN* expression, as assessed by qRT-PCR analysis. These 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. By contrast, when *PTEN* was knocked down, 20E could not inhibit insulin-induced Akt phosphorylation (Fig. 7D). These data confirmed that 20E inhibits Akt phosphorylation by increasing PTEN expression.

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

We found a FoxOBE motif (5′-TTGTTTAA-3′) in the upstream region (−845 to −838 bp, relative to the ATG) of *BrZ7* (Fig. S3). The C in the conserved FoxOBE 5′-TTGTTTAC-3′ (Eijkelenboom and Burgering, 2013) was replaced by A in FoxOBE of *BrZ7*, but the highly conserved FoxOBE common core sequence (5′-AAACA-3′) in the complementary strand (Barthel et al., 2005) was retained.

Given that the expression of *BrZ7* was suppressed after *FoxO* knockdown in the larval experiments, we examined FoxO regulation of *BrZ7* transcription. FoxO-GFP-His was overexpressed in HaEpi cells by transfection of plasmid pIEx-4-FoxO-GFP-His. In a chromatin immunoprecipitation (ChIP) assay, a small amount of the quantitative (q) RT-PCR product of the FoxOBE-containing DNA fragment was detected in immunoprecipitates of 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 immunoprecipitates of 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 bp downstream of the FoxOBE-containing DNA fragment. The data showed that FoxO did not bind to this fragment (Fig. 8B). This showed that FoxO binds specifically 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′-TTTTTGCATTGTTTAAAATAGCAGC-3′) and the purified FoxO-GFP-His protein from the nuclei of 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

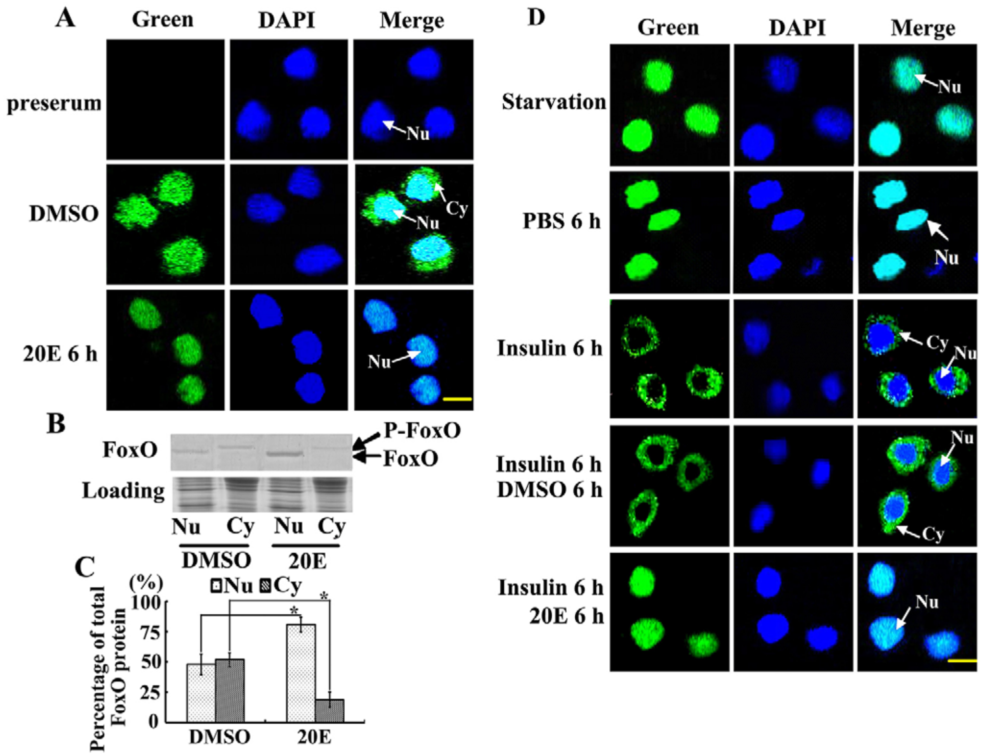

Fig. 5. 20E regulates FoxO nuclear localization and inhibits FoxO phosphorylation in HaEpi cells. (A) FoxO translocates to the nucleus after 20E induction in Grace's medium with 10% FBS. Cells were incubated with 1 µM 20E for 6 h. Green fluorescence indicates FoxO (immunodetection as in Fig. 4A). Blue, nuclei (DAPI). (B) 20E inhibits FoxO phosphorylation (P-FoxO), as assessed by western blotting via 7.5% SDS-PAGE. The same proteins were separated and stained with CBB after SDS-PAGE to provide a protein loading control. Cells were incubated with 1 µM 20E for 6 h in Grace's medium with 10% FBS. Control cells received the same amount of DMSO. (C) Statistical analysis of the data in B. *n*=3. **P*<0.05 (Student's *t*-test). Error bars indicate the mean±s.d. of three independent biological experiments. (D) 20E induces 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 for 6 h plus DMSO or insulin plus 1 µM 20E for another 6 h in Grace's medium without FBS. Scale bars: 20 µm.

detected using 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, whereas 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′-TTTTTGCAATGCATGCAATA-GCAGC-3′) was used as competitive inhibitor. Binding of FoxO to the digoxigenin-labeled FoxOBE probe was detected, and this was competed with the unlabeled FoxOBE probe but not with unlabeled FoxOBE-M (Fig. 8E,F). These results indicated that FoxO binds to the FoxOBE of *BrZ7* to regulate *BrZ7* transcription directly.

20E regulates a 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 expression of *FoxO*, *BrZ7* and *CPA* (Fig. 9A,B). *FoxO* knockdown repressed *BrZ7* and *CPA* expression, but did not repress *EcRB1* and *USP1* expression (Fig. 9C). *BrZ7* knockdown repressed *CPA* expression, but did not repress that of *FoxO* (Fig. 9D). These results identified the cascade of gene expression during 20E induction: 20E, via EcRB1 and USP1, upregulates FoxO expression, which in turn directs BrZ7 expression, leading to CPA expression.

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 FoxO in the fat body cells of *D. melanogaster* to counteract insulin activity (Colombani et al., 2005). However, the mechanism by which 20E counteracts insulin activity is not well understood. Br (or Br-C) is a metamorphic inducer in the 20E pathway (Erezyilmaz et al., 2006), but the mechanism by which 20E upregulates Br expression had not been determined. The present study revealed that 20E upregulates PTEN and FoxO expression, and inhibits Akt and FoxO phosphorylation, resulting in the nuclear localization of non-phosphorylated FoxO. In the nucleus, FoxO binds to FoxOBE in the 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 phosphoinositide-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). By contrast, PTEN degrades the phosphate from PIP3 to produce PIP2, which cannot attract Akt to the cell membrane; therefore, Akt cannot be phosphorylated (Maehama and Dixon, 1998), and 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

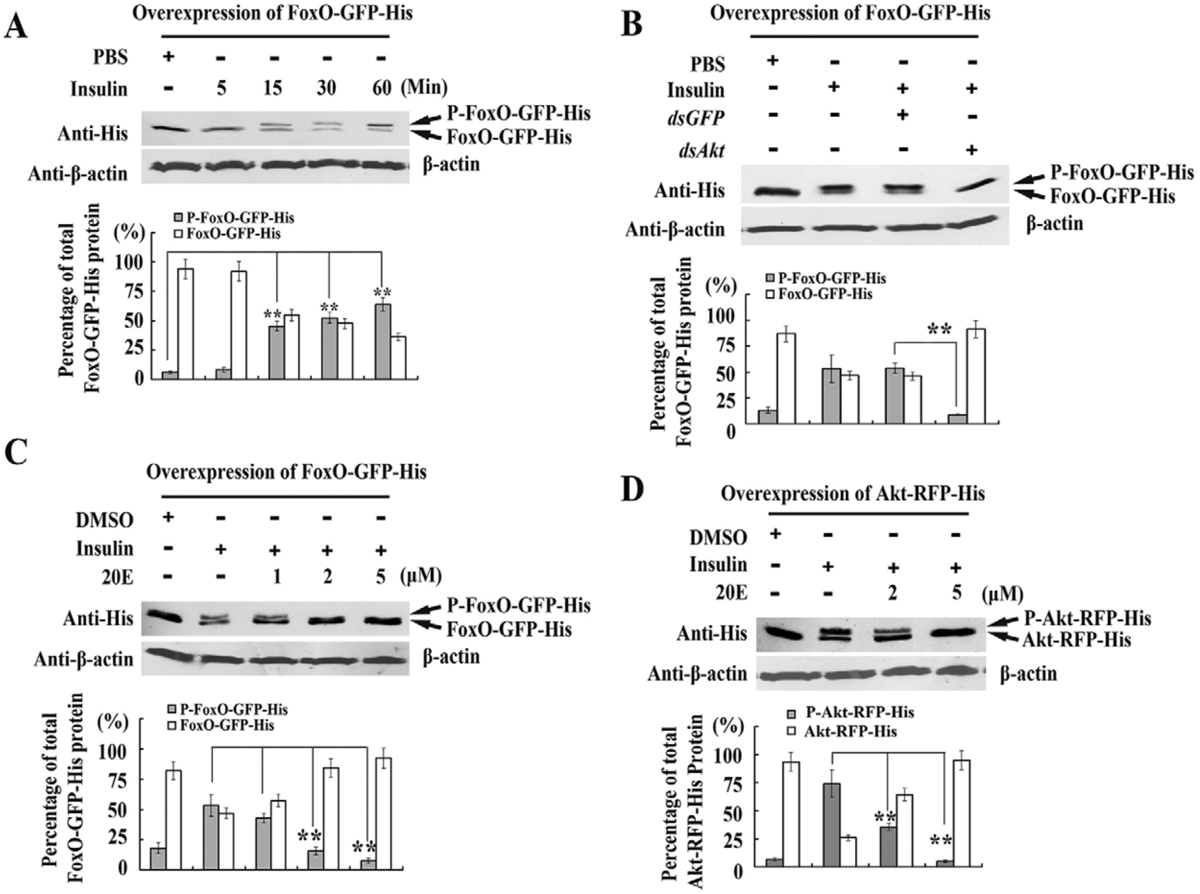

Fig. 6. 20E inhibits FoxO phosphorylation by repressing Akt phosphorylation. (A) Insulin induces FoxO phosphorylation in HaEpi cells. The cells were transfected with pIEx-4-FoxO-GFP-His plasmid for 48 h, and then treated with insulin (2.5 μg/ml) for 5, 15, 30 and 60 min, respectively, before western blotting. (B) Insulin, via Akt, induces 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 and then assessed by western blotting. (C) 20E inhibits 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 represses 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. For all western blot analyses in this figure, 7.5% SDS-PAGE was used. *n*=3. $^{**}P<0.01$ (Student's *t*-test). Error bars indicate the mean±s.d. of three independent biological experiments.

cytoplasm. However, 20E upregulates PTEN expression, which inhibits Akt and FoxO phosphorylation to induce FoxO nuclear translocation. 20E upregulates *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 (ILP8) coordinates growth and maturation (Colombani et al., 2012). The expression and secretion of ILP8 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 PG 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 data 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 of the old cuticle, might 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 signaling pathway

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

In the upstream region (−933 bp) of *H. armigera BrZ7*, no EcRE was predicted by searching using the conserved sequence (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 −4950 bp and −3480 bp EcREs in the upstream distal promoter in *B. mori* (Nishita, 2014). *Br*

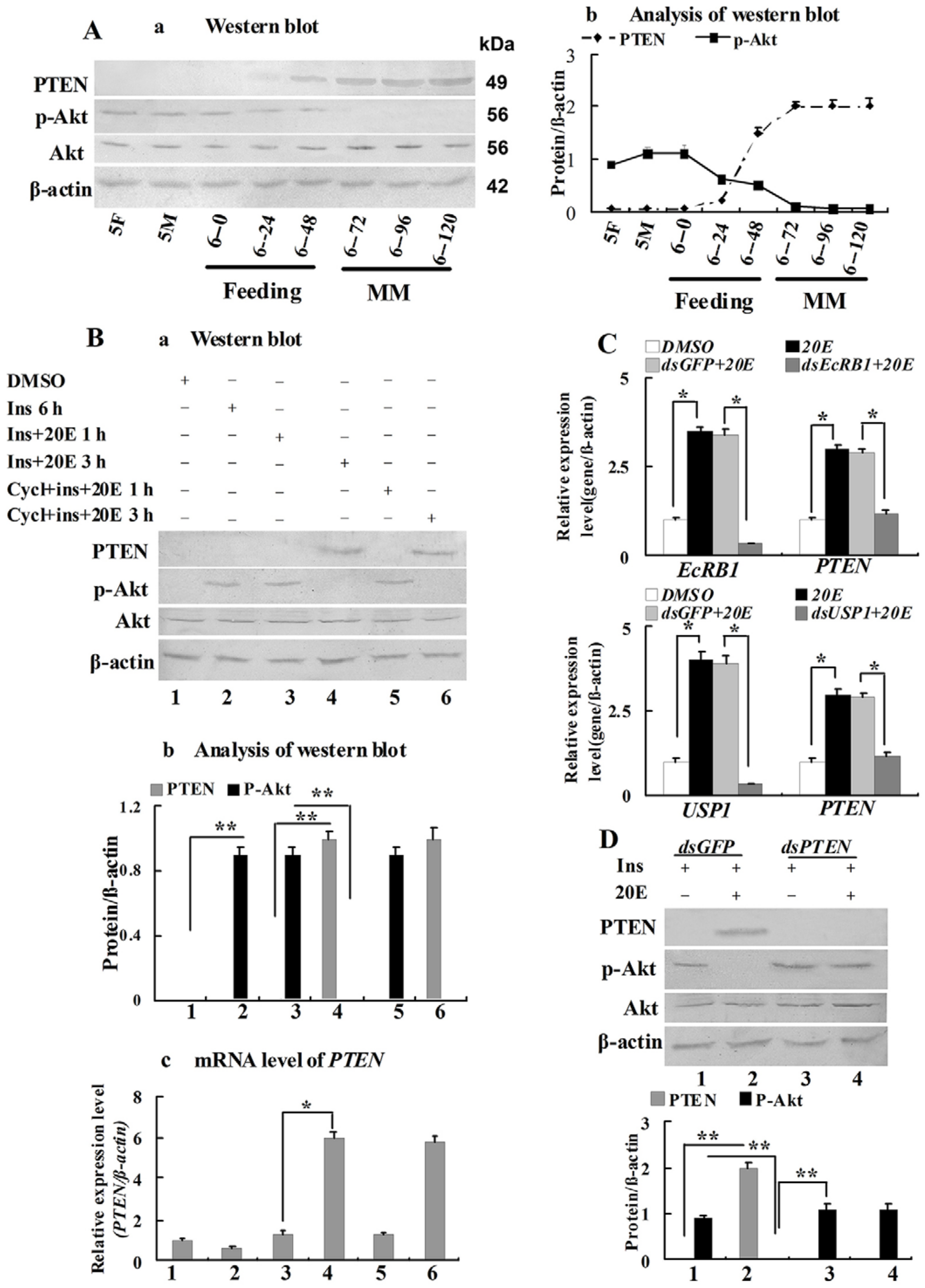

Fig. 7. 20E upregulates PTEN expression to repress Akt phosphorylation. (Aa) Western blot analysis of expression levels of PTEN and phosphorylated Akt in the integument during larval development (stages as in Fig. 1A,B), as detected by antibodies to human PTEN, human phosphorylated Akt, *H. armigera* Akt and *H. armigera* β-actin. (Ab) Quantification of the data in Aa. (B) Hormone induction of PTEN expression and Akt phosphorylation. (Ba) Western blot. Lanes: 1, DMSO as solvent control; 2, insulin (2.5 µg/ml) for 6 h; 3 and 4, insulin (2.5 µg/ml for 6 h) 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 for 6 h) and 20E (1 µM) for another 1 or 3 h, respectively. Ins, insulin; Cycl, cycloheximide. (Bb) Quantitative analysis (using ImageJ) of the PTEN expression and phosphorylated Akt data in Ba. (Bc) qRT-PCR analyses showing the effect of cycloheximide on the mRNA levels of *PTEN* after the same treatments as in Ba. (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) and/or 20E (1 µM for 6 h). (D) *PTEN* knockdown maintained Akt phosphorylation under 20E treatment. The cells were treated with dsRNA (1 µg/ml for 12 h) and insulin (2.5 µg/ml) with or without 20E (1 µM) for another 6 h. Quantitative analysis (using ImageJ) of the western blot is shown beneath. *$P<0.05$, **$P<0.01$ (Student's *t*-test), based on three independent experiments. Error bars indicate the mean±s.d. of three independent biological experiments.

transcription is also upregulated by JH 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 upstream region (−933 bp) of *H. armigera BrZ7*. In addition to transcriptional regulation, BrZ7 is also regulated post-translationally. BrZ7 is phosphorylated upon JH induction but is not phosphorylated upon 20E induction (Cai et al., 2014b). The BrZ7 protein level and its activity as a transcription factor are 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 play a key role in molting by promoting proteolysis during apolysis (Samuels and Paterson, 1995). CPA in the molting fluid participates in apolysis 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 leads to molting failure because of the suppression of *BrZ7* and *CPA* expression. *BrZ7* knockdown did not repress *FoxO* expression but did repress *CPA* expression, indicating that FoxO regulates CPA expression via BrZ7. This finding revealed that FoxO participates in 20E-induced proteolysis during molting by regulating *BrZ7* and *CPA*

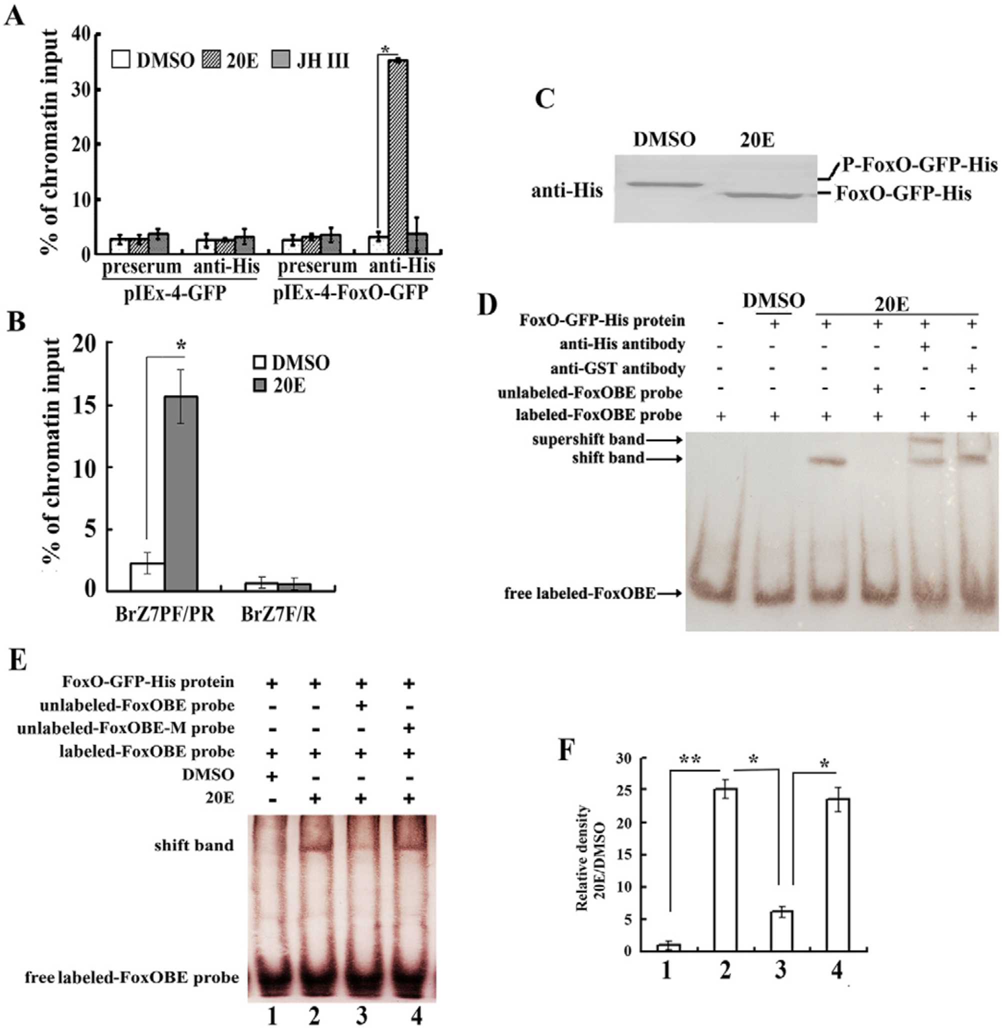

Fig. 8. FoxO binds to the FoxOBE in the upstream region of *BrZ7*. (A) ChIP assay of FoxO binding to the upstream region of *BrZ7* using primers PF/PR. Cells were transfected with pIEx-4-FoxO-GFP-His or pIEx-4-GFP-His for 48 h, and then treated with DMSO, 1 μM 20E or JH III. An anti-His antibody was 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 upstream region of *BrZ7*, using primers BrZ7F/BrZ7R to exclude non-specific FoxO binding to DNA. (C) Western blot showing the phosphorylation status of FoxO under DMSO control 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 (FoxOBE-M) 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. *$P<0.05$, **$P<0.01$ (Student's *t*-test). Error bars indicate the mean±s.d. of three independent biological experiments.

expression. CPA is also highly expressed during metamorphosis in *H. armigera* (Sui et al., 2009); therefore, FoxO could also regulate metamorphic molting. However, any effect of *FoxO* knockdown on metamorphic molting could not be examined in our study 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 the transcription of a series of genes in humans (Huang and Tindall, 2011). In *Drosophila*, FoxO mediates the reduction in cell number associated with reduced insulin signaling. *Drosophila* that are homozygous for *foxo* null alleles are viable and of normal size; however, they are sensitive to oxidative stress (Jünger et al., 2003). The knockdown of *FoxO* in *H. armigera* resulted in the larvae failing to molt and shed their old cuticle, and they died before entering the next stage. The differing results obtained from *Drosophila* and *Helicoverpa* might be attributed to these being different orders of insects and the depletion of FoxO occurring at different developmental stages.

Conclusions

Insulin induces Akt phosphorylation; in turn, Akt induces FoxO phosphorylation and cytoplasmic localization, which allows larval growth to produce more 20E. The high level of 20E then upregulates

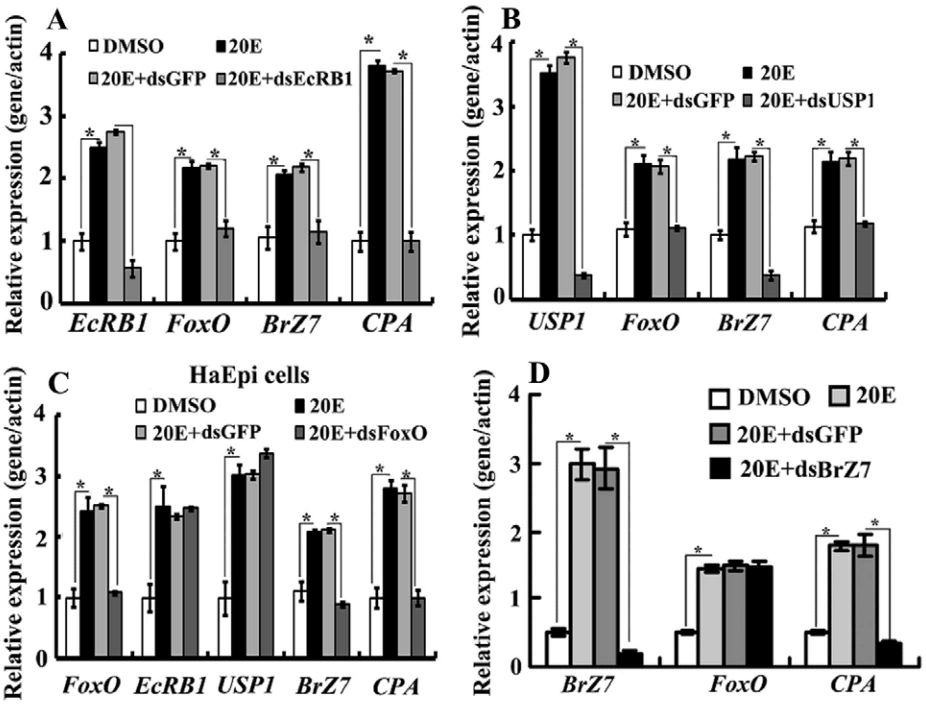

Fig. 9. Cascade of gene expression in 20E regulation in HaEpi cells. (A,B) 20E upregulates *FoxO* expression via EcRB1 and USP1. (C) 20E regulates *BrZ7* and *CPA* expression via FoxO. (D) 20E regulates *CPA* expression via BrZ7. The cells were incubated with dsRNA (4 µg/ml) for 10 h and induced by 1 µM 20E for 6 h. The control samples received the same amount of *dsGFP*. Total RNA was isolated for qRT-PCR analysis. *$P<0.05$, (Student's *t*-test), based on three independent biological experiments. Error bars indicate the mean±s.d. of three independent biological experiments.

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 an artificial diet using previously described methods (Zhao et al., 1998).

Expression of recombinant FoxO and preparation of antiserum

A fragment (amino acids 82-921) of FoxO was expressed in *Escherichia coli* BL21 (DE3) from the pET30a (+) vector (Novagen). The recombinant FoxO proteins were purified by 12.5% SDS-PAGE. The purified recombinant FoxO protein was used to prepare rabbit polyclonal antiserum as previously described (Cai et al., 2014b). Details are provided in the supplementary Materials and Methods.

Western blot

Proteins from various tissues were extracted in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.5) with 1 mM phenylmethanesulfonyl fluoride (PMSF). 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. Detection was by protein-specific polyclonal antibodies (1:200 in blocking solution) and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000 in blocking solution). For details, see the supplementary Materials and Methods.

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) with a CFX96 real-time system (Bio-Rad) and the primers listed in Table S1. The relative expression levels of the genes were quantified using *H. armigera β-actin* as an internal control. Experiments were repeated three times. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Liu et al., 2013).

Hormonal regulation

The epidermal cell line (HaEpi) was established from the fifth instar larval integument of *H. armigera* in our library laboratory (Shao et al., 2008). Cells were frozen from the fifth passage primarily in Grace's medium (20% FBS) and 10% dimethyl sulfoxide (DMSO), and were then cultured and maintained for experiments. The culture method was as described previously (Liu et al., 2011). At confluence ranging from 70% to 90%,

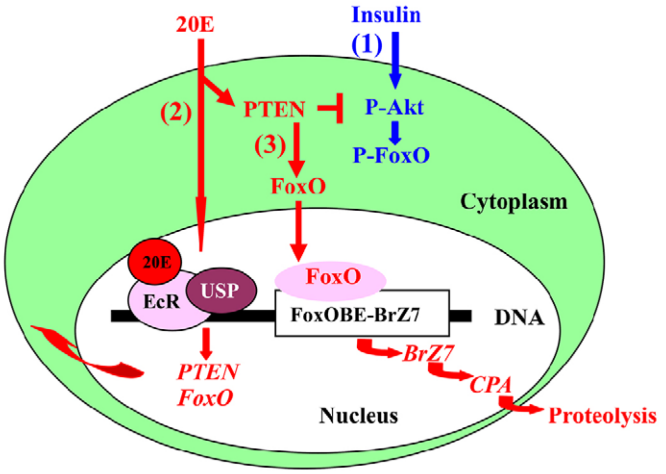

Fig. 10. Schematic of 20E activation of FoxO to counteract insulin activity. (1) Insulin induces Akt phosphorylation, which regulates FoxO phosphorylation and cytoplasmic localization. (2) 20E, through EcRB1 and USP1, upregulates PTEN and FoxO expression. (3) PTEN represses the phosphorylation of Akt,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

the cells were treated with 20E or insulin. Controls were treated with the same amount of DMSO. Sixth instar 6 h larvae were injected with 20E or JH III (500 ng/larva) or with DMSO as control. 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 using primers RNAi-F/R and GFP-RNAi-F/R (Table S1). dsRNA was synthesized using the MEGAscript RNAi Kit (Ambion) according to the manufacturer's instructions. 1 μg *dsFoxO* was injected into fifth instar 12 h larvae. Controls were treated with the same amount of *dsGFP*. Total RNA was extracted to detect the effects of RNAi 2 days 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 (PFA) overnight at 4°C. The tissues were dehydrated with an ethanol gradient and embedded in paraffin. The paraffin sections were cut into 7 μm 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 sections 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 goat anti-rabbit Alexa Fluor 488 or 568 (1:1000 in 2% BSA) secondary antibody in the dark for 2 h at room temperature and stained with DAPI (AnaSpec). For further details, see the supplementary Materials and Methods.

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 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% PFA for 15 min and washed three times with PBS. The cells were then permeabilized in PBS containing 0.2% Triton X-100 for 10 min and blocked with 2% BSA in PBS for 30 min. The anti-FoxO antibody (1:200) was added to the cells overnight at 4°C. The cells were then 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 with the primary antibody replaced with preserum.

RNAi in the HaEpi cell line

Transient transfection was performed using RNAfectin (Tiangen, Beijing, China) according to the manufacturer's instructions. The HaEpi cells were cultured in 1 ml Grace's medium with dsRNA and RNAfectin without FBS at 80% confluence. The final concentrations of dsRNA and RNAfectin transfection reagent were 2 μg/ml and 4 μg/ml, respectively. After 12 h, the cells were replenished with fresh medium with FBS and 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 ChIP

The ORF of *FoxO* was inserted into vector pIEx-4-GFP-His [we constructed the plasmid by inserting a *GFP* sequence into the pIEx-4-His plasmid (Invitrogen)] 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 with anti-FoxO antibodies. DNA was purified using phenol/chloroform extraction and ethanol precipitation to use as templates for qRT-PCR. The upstream region of *BrZ7* was cloned using the genome walking method. BrZ7PF/PR and BrZ7F/R primers are listed in Table S1. The input was the amount of chromatin DNA before immunoprecipitation. The data were calculated according the following formula: percentage of chromatin $\text{input}=100\times2^{-\{\text{Ct}}\text{ChIP}^{-[\text{Ct}}\text{Input}^{-\log}2^{(\text{input dilution factor})]\}}$, where Ct_{ChIP} is the Ct of qRT-PCR from the antibody precipitate, Ct_{Input} is the Ct of qRT-PCR before immunoprecipitation, and the input dilution factor is (fraction of the input chromatin saved)$^{-1}$. For details, see the supplementary Materials and Methods.

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 or the same volume of DMSO as control. 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 6.5% PAGE. The samples were then transferred into a nylon membrane and the DNA band detected using phosphatase-labeled anti-Dig antibody. For further details, see the supplementary Materials and Methods.

Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors contributed equally to this work. M.-J.C. performed and analyzed the experiments in Figs 4, 5, 8 and 9. W.-L.Z. performed and analyzed the experiments in Figs 5 and 7. Y.-P.J. performed and analyzed the experiments in Figs 6 and 8. Q.S. performed and analyzed the experiments in Figs 1-4. X.-Q.Z. performed and analyzed the experiments in Fig. 7. J.-X.W. and X.-F.Z. conceived and coordinated the study, edited the paper and drafted Fig. 10. All authors reviewed the results and approved the final version of the manuscript. All co-authors have checked and confirmed their contribution statement.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.128694/-/DC1

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