

Supplementary Materials and Methods

Sequence analysis

The full sequence of FoxO was obtained from transcriptome sequencing of a cDNA library of *H. armigera* epidermal cell line. A pair of specific primers, *FoxO-seq-F* and *FoxO-seq-R* (Supplementary files: Table S1), was used to amplify the open reading frame (ORF). The cDNA and encoded protein were analyzed by BLASTX (<http://www.ncbi.nlm.nih.gov/>), ExPASy (<http://www.expasy.org/tools/>), and SMART (<http://smart.embl-heidelberg.de/>). DNAMAN software was used to perform sequence alignment.

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 (200 µg) was mixed with the same volume of Freund's complete adjuvant (Sigma-Aldrich Corporation, St. Louis, MO, USA) for rabbit injection. After 3 weeks, approximately 500 µg of proteins were mixed with the same volume of Freund's incomplete adjuvant for injection. The serum was collected 2 weeks after injection, and the specificity of the antiserum was analyzed by western blotting. The secondary antibody was alkaline phosphatase-labeled goat anti-rabbit IgG (H+L) (Zhongshan, Beijing, China).

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 membrane was shaken for 1 h in a blocking solution (2% nonfat dry milk in TBS) and subsequently incubated with specific polyclonal antibodies (1:200 in blocking solution) overnight. The anti-*Homo*-PTEN (wl01901) and anti-*Homo*-phosphorylated-Akt (wlP001a) antibodies were purchased from Wanleibio (Shenyang, China). The polyclonal antibodies against *H. armigera*-Akt, FoxO, CPA and beta-actin were produced in our laboratory. After being washed three times with TBST (0.02% Tween 20 in TBS) for 10 min, the membrane was incubated in alkaline phosphatase-conjugated goat-anti-rabbit IgG (1:10,000 in the blocking solution) for 3 h. The membrane was washed three times with TBST for 10 min each and once in TBS for 10 min. The signal was detected using 10 mL TBS adding with 45 µL 5% nitroblue tetrazolium (NBT) and 35 µL 5% 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Protein overexpression and chromatin immunoprecipitation (ChIP)

The ORF of FoxO was inserted into vector pIEx-4-GFP-His to express FoxO (with C-terminal GFP and histidine tags). Transcription was driven by the AcNPV-derived hr5 enhancer and the immediate early promoter IE1. HaEpi cells were incubated in Grace's

medium with 10% FBS. Before transfection, cells were pre-incubated in Grace's medium (without FBS) for 1 h. Afterward, 2 μ g pIEx-4-FoxO-GFP-His and 2 μ g DNAfectin transfection reagent (Tiangen, Beijing, China) were suspended in 50 μ L Grace's medium, incubated for 20 min and added into the medium. After 12 h, the cells were refreshed in Grace's medium with 10% FBS for 48 h. The controls were transfected with the same amount of pIEx-4-GFP-His plasmids. The cells were then incubated in 1 μ M 20E or JH III for 3 h. The control group received the same volume of DMSO. The cells were cross-linked with 0.5% formaldehyde at 37°C for 10 min and incubated in 0.125 M glycine at room temperature for 10 min to stop the reaction. After being washed twice with ice-cold 1 \times PBS, the cells were harvested and resuspended in SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and sonicated to yield DNA fragments with lengths ranging from 200 bp to 1000 bp. After centrifugation, the lysates were precleared with protein A resin at 4°C for 1 h and immunoprecipitated with anti-His antibody at 4°C overnight. Protein-DNA complexes immunoprecipitated by anti-FoxO antibodies were incubated in protein A resin at 4°C for 2 h. The complexes were washed once with low-salt wash buffer (0.1% SDS, 1.0% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.0, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1.0% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), LiCl wash buffer (10 mM Tris-HCl, pH 8.1, 0.25 M LiCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate) and twice with TE buffer (10 mM Tris-HCl, pH 8.1, 1 mM EDTA). Bound proteins were eluted with an elution buffer (1% SDS, 0.1 M NaHCO₃). DNA-protein cross-links were reversed at 65°C overnight, and then incubated with RNase and proteinase K. 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 genomic DNA was isolated using a MagExtractor genomic DNA purification kit (Toyobo, Osaka, Japan). 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 = $100 \times 2^{-(Ct_{[ChIP]} - (Ct_{[Input]} - \log_2(\text{Input Dilution Factor})))}$. Ct_{ChIP}: the Ct of qRT-PCR from anti-body precipitate, Ct_{Input}: the Ct of qRT-PCR before immunoprecipitation. Input Dilution Factor = (fraction of the input chromatin saved)⁻¹.

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 μ M 20E; 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 μ g/mL Leupeptin, 20 μ g/mL Aprotinin, 125 μ M DTT, 1 mM PMSF). The nuclear proteins were isolated with extraction buffer (500 mM KCl, 25 mM HEPES pH 7.8, 10% glycerol, 10 μ g/mL Leupeptin, 20 μ g/mL Aprotinin, 125 μ M DTT, 1 mM PMSF). FoxO-GFP-His protein

was purified using anti-His antibody-CNBr-activated Sepharose 4B (60 mg; Amersham Biosciences AB, Uppsala, Sweden). Approximately 5 μ g (5 μ L) of purified protein in binding buffer (Beyotime Institute of Biotechnology, Shanghai, China) was incubated in 100 fmol digoxigenin (Dig)-labeled FoxO binding element (FoxOBE) probe (Sangon Company, Shanghai, China). In the competition experiments, a 100-fold excess of unlabeled probe was pre-incubated with the purified protein for 10 min. An unlabeled mutational probe (FoxOBE-M probe) was also pre-incubated to identify the specificity of the FoxO binding element. A Dig-labeled probe was then added for another 20 min at room temperature. For the supershift experiment with the antibody, 1 μ L of anti-His antibody was added to the proteins and incubated in an ice bath for 10 min before the labeled probe was added. The non-specific anti-GST antibody was used as the nonspecific antibody negative control. The reaction was applied to a 6.5% polyacrylamide gel prepared with TAE buffer (890 mM Tris-acetic acid, 20 mM EDTA) at 80 V in running buffer (445 mM Tris-acetic acid, 10 mM EDTA). The samples were then transferred into a nylon membrane (IMMOBILON-NY⁺, Millipore, Milford, MA, USA). The membrane was blocked for 30 min and then incubated with phosphatase-labeled anti-Dig antibody (1:10,000 in blocking solution; Roche, Upper Bavaria, Germany). After 1 h, the signal was obtained using NBT and BCIP according to previously published western blot methods.

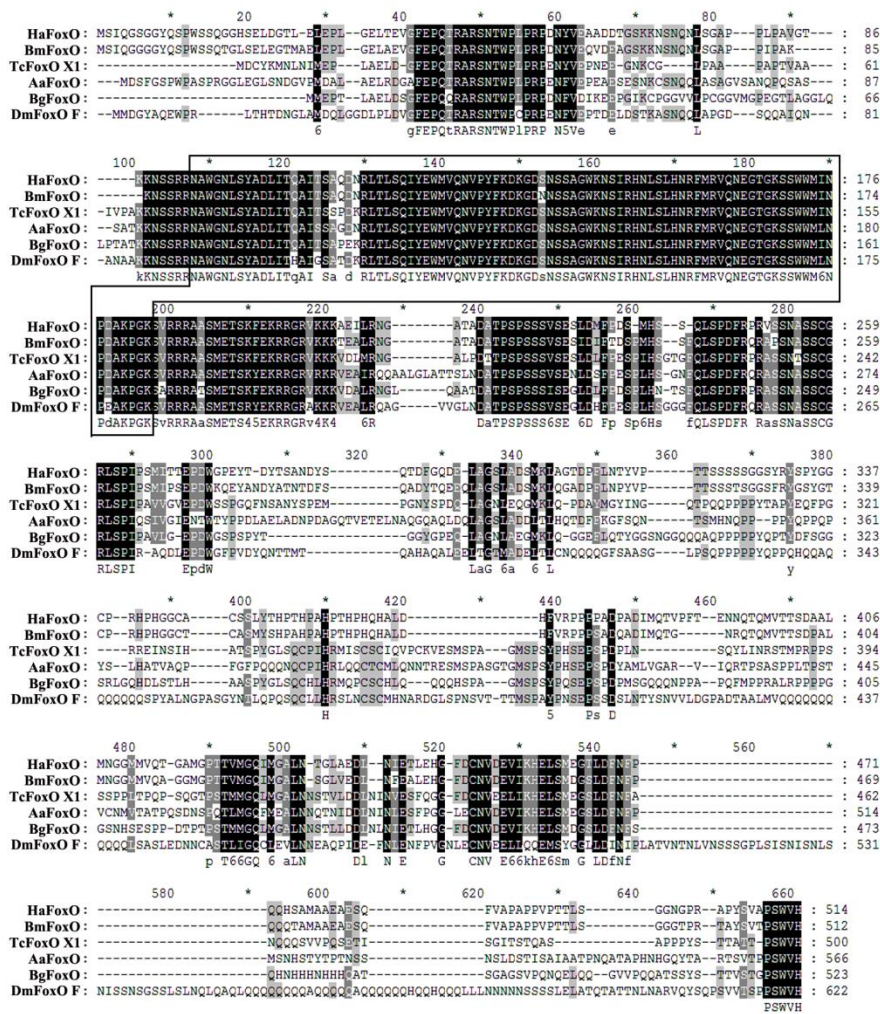
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A E I L R N G A T A D A T P S P S S S V 227
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S E S L D M F P D S X M H S S X Q L S P 247
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D F R P R V S S N A S S C G R L S P I P 267
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L D F N F P Q Q H S A M A A E A E S Q F 487
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V A P A P P V P T T L S G G N G P R A P 507
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Y S V A P S W V H - 516
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Figure S1. Full-length cDNA sequence and predicted amino acid sequence of FoxO. The isoelectric point (pI) is 5.7, and the molecular weight is 55.8 kDa. The double underlined sequences correspond to the putative phosphorylation sites. The conserved forkhead domain (94-183 aa) is underlined.

A.



B.

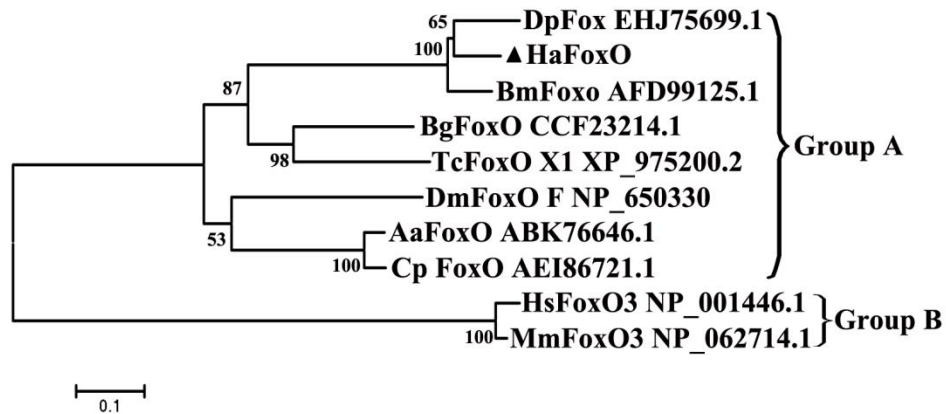


Figure S2. A. Multiple alignments of FoxO from different species. Ha, *Helicoverpa armigera*; Bm, *Bombyx mori*; TcFoxO X1, FoxO isoform X1 from *Tribolium castaneum*; Aa, *Aedes aegypti*; Bg, *Blattella germanica*; DmFoxO F, FoxO isoform F from *Drosophila melanogaster*. **B.** Phylogenetic tree analysis of FoxO from different species. DpFox, Fox from *Danaus plexippus*; HsFoxO3, FoxO3 from *Homo sapiens*; MmFoxO3, FoxO3 from *Mus musculus*.

CGACGGCCCTGGCTGGTCCTGTTAATAACAGGCTTTGCTCTTTTTTACCAGTAG
GAGTCAAATTTCAACTGACTGTATTTTGCATTTGTTTAAATAGCAGCAAACATTG
TTCTTAGCTATTGTAGGAAATATCATGTAGTTTATTAATAAATAAAAGAAAGTGATA
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AGATTATTCGACAGAAACCATAGGGATG

Figure S3. The 5' regulatory region of *BrZ7*. The underlined sequence corresponds to the FoxO binding sequence. The sequence for CHIP is in shadow. Doubl underlined ATG is the start code of the *BrZ7*.

Table S1. Primers for qRT-PCR and RNA interference

Primer names	(5'-3') nucleotide sequence
Gene cloning	
FoxO-seq-F	atgtctatacagggcagcgg
FoxO-seq-R	cgaaggttcggatggtatgtct
FoxO-EXP-F	tactcagaattcttggaaaccttgggag
FoxO-EXP-R	tactcactcgagctacatggaatcggcgagaga
Oeverexpression	
FoxoplExF	tactcaagatctatgtctatacagggcagcgg
FoxoplExR	tactcagtcgacgtggaccaggagggcgac
RNA	
GFP-RNAi-F	gcgtaatacactcactataggtggccaattctctggaac
GFP-RNAi-R	gcgtaatacactcactataggcttgaagttgacctgatgcc
FoxO-RNAi-F	gcgtaatacactcactataggcaagacaacagactcacg
FoxO-RNAi-R	gcgtaatacactcactataggttccgaagtccgtttg
EcRB1-RNAi-F	gcgtaatacactcactataggcgtggtataacaacggagg
EcRB1-RNAi-R	gcgtaatacactcactataggagctggagacaactcctcac
USP1-RNAi-F	gcgtaatacactcactataggaaggctcctggaacgaa
USP1-RNAi-R	gcgtaatacactcactataggataggcggcgctggtg
qRT-PCR	
FoxO-qF	ggctccaagaagaactcca
FoxO-qR	cattctgaacctcactcg
EcRB1-F	cgctggtataacaacggagga
EcRB1-R	agctggagacaactcctcacg
USP1-F	aagggtcctggaacgaa
USP1-R	ataggcggcgctggtg
PTEN-qRTF	ttatgggtattgctgag
PTEN-qRTR	taggctaagtccaggtga
BrZ7-F	atggctgatcaattctgttta
BrZ7-R	gttcggatgaagagaaatcttc
CPA-F	ggaagcagtatcatagactgg
CPA-R	atcttgaagctccttgcctc
β -actin-F	cctggtattgctgaccgtatgc
β -actin-R	ctgttgaaggtggagaggaa
ChIP assay	
BrZ7PF:	gtcctgtaataacaggct
BrZ7PR:	aataacgcctcttttca
BrZ7-F	atggctgatcaattctgttta
BrZ7-R	gttcggatgaagagaaatcttc