Supplementary Materials and Methods

Cloning of the orange-spotted grouper foxo3a and foxo3b cDNAs

The initial cDNA fragments for foxo3a and foxo3b were amplified with nested PCR from the ovary cDNA. The primers were EcFoxo-F1 and EcFoxo-R1 for the first round and EcFoxo-F2 and EcFoxo-R1 for the second round of amplification. These primers were listed in Supplementary Table 1. The ovary cDNA was reverse transcribed from the ovary total RNA, by the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to manufacturer’s instructions using primer AP. PCR was performed in a 25-μl final volume containing 2.5 μl 10x Taq Buffer, 2.5 mM MgCl₂, 0.2 mM dNTP, 0.4 μM of each primer, 1.25 U Fermentas Taq DNA Polymerase (Fermentas), and 1 μl of ovary cDNA. After an initial 3 min denaturing step at 94 °C, 35 cycles of amplification were performed with 0.5 min at 94 °C, 0.5 min at 53 °C, and 2.0 min at 72 °C, and then followed by a final extension for 30 min at 72 °C. The target PCR band (about 450 bp) was gel-purified using Gel Extraction System (Omega Bio-tek, GA, USA) and cloned into the pTZ57R/T Vector (Fermentas). Eleven clones were sequenced in both directions with forward and reverse universal primers using the Bigdye-Terminator kit and an ABI Prism 3730XL DNA sequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA, USA), and two different sequences corresponding to either foxo3a or foxo3b of teleosts were obtained, respectively. Then the 3’-ends of cDNAs were obtained with the RACE method by nested PCR. The primers were EcFoxo3a-F3 and AUAP for the first round and EcFoxo3a-F4 and AUAP for the second round of amplification of foxo3a, and EcFoxo3b-F3 and AUAP for the first round and EcFoxo3b-F4 and AUAP for the second round of amplification of foxo3b. These primers were
listed in Supplementary Table 1. The cycling conditions were the same as the above except that annealing temperature was 55 °C. The target PCR products were processed and analyzed as above.

For the 5’ RACE, the above synthesized single strand cDNA were first purified by a E.Z.N.A. DNA Probe Purification kit (Omega Bio-tek) and modified by tailing reaction using Terminal Deoxynucleotide Transferase (TdT) and dCTP (Takara, Dalian, China). Then, 5’ ends of cDNAs were amplified with nested PCR, using primers EcFoxo3a-R3 and AAP for the first round and EcFoxo3a-R4 and AUAP for the second round of amplification of *foxo3a*, and EcFoxo3b-R3 and AAP for the first round and EcFoxo3b-R4 and AUAP for the second round of amplification of *foxo3b*. These primers were listed in Supplementary Table 1. The PCR cycling conditions were the same as the above except that annealing temperature was 55 °C. The target PCR products were processed and analyzed as above, and the full-length cDNA sequences were obtained by combining the sequences of the initial fragment, and 3’- and 5’-ends.

**RT-PCR analysis of foxo3a and foxo3b tissue distribution patterns**

Total RNA isolated from tissues was first treated with DNase I (1 U/μl) to remove any genomic DNA contamination. Then 1 μg total RNA was reverse transcribed with oligo(dT)$_{18}$ primers using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. The integrity of all RNA samples was verified by the successful amplification of β actin (AY510710).

The first-strand reaction (1 μl) was amplified for each target gene using the TGRADIENT thermocycler (Biometra GmbH, Goettingen, Germany). PCR was performed in
a 20-μl final volume containing 2.0 μl 10×Taq buffer, 2.0 mM MgCl₂, 0.2 mM dNTP, 0.4 μM of each primer, and 1.25 U Taq DNA Polymerase (Fermentas). Water was used as a negative control in the RT-PCR. The reaction mixture was heated at 94 °C for 3 min, followed by 34 cycles for foxo3a and foxo3b, and 25 cycles for β actin. The cycling conditions were 94 °C for 0.5 min, 60 °C for 0.5 min, 72 °C for 1 min, with a final extension at 72 °C for 7 min. The primers were EcFoxo3a-RT-F3 and EcFoxo3a-RT-R3 for foxo3a, EcFoxo3b-RT-F1 and EcFoxo3b-RT-R1 for foxo3b, and Ecβactin-RT-F1 and Ecβactin-RT-R1 for β actin, which generated PCR products of 459 bp, 442 bp, and 530 bp, respectively. These primers were listed in Supplementary Table 1, and the primer sets were designed to span the intron, respectively. The PCR products were separated on 2.0% agarose gels, and stained with ethidium bromide (0.5 μg/ml). The gel images were captured on the Biorad GelDoc 2000 (Bio-Rad, CA, USA). At least three independent assays were performed to confirm the mRNA expression patterns in tissues, and the authenticities of the amplicons were confirmed by sequencing.

**Cell culture, transient transfection, and luciferase assay**

TM4 (mouse Sertoli cell line) cells were grown in DMEM/F12 (Invitrogen) supplemented with 2.5% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and 5% horse serum (Gibco). COS-7 (Cercopithecus aethiops SV40 transformed kidney cell line) cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Gibco). The cell culture medium contained 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), and cells were maintained at 37°C in a humidified incubator under 5% CO₂ and used for transfection study when they were between passage 10 to 20.
Plasmids for transfection were prepared from overnight bacteria cultures using the PureLink™ HI-Pure Plasmid DNA Purification Kit (Invitrogen) according to the manufacturer’s instruction. Twenty four hours prior to transfection, cells were plated on 24-well plates \(10^5\) cells/well, and transiently co-transfected with promoter reporter constructs (280 ng/well), an internal control vector pRL-TK (20 ng/well; Promega Life Science, Madison, WI, USA), and a Foxo3a- or Foxo3b- expression vector (100 ng/well) using FuGENE® HD Transfection Reagent (Roche, Mannheim, Germany; 1 μl/well) according to the manufacturer’s instruction. The Firefly and Renilla luciferase activities were measured 48 h later using the Dual-Luciferase Reporter Assay System (Promega) and TECAN Infinite M200 (TECAN, Switzerland). The Firefly luciferase data were corrected for transfection efficiency with Renilla luciferase activity. Each transfection reaction was carried out in triplicates, and the experiments were repeated at least three times.

**Western blot analysis**

Tissue homogenates (500 μg) were separated on a 12% SDS-PAGE gel and transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane (Millipore, CA, USA) by electroblotting. The membrane was then blocked with 5% non-fat milk powder in 10 mM PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO₄, 2 mM KH₂PO₄) at 4 °C overnight. The anti-Foxo3a or anti-Foxo3b antiserum was pre-adsorbed for 4 h at 4 °C with extracts of *E. coli* BL21 (DE3) bacteria which were transformed with the empty vector pET-32a and induced by IPTG. To examine the specificity, the anti-Foxo3a antiserum or anti-Foxo3b antiserum was further pre-adsorbed with the full-length Foxo3a (EcFoxo3a) or Foxo3b (EcFoxo3b) for the negative control, respectively. EcFoxo3a and EcFoxo3b were nuclear
extracts of COS-7 cells transfected with Foxo3a and Foxo3b expression vectors derived from pcDNA3.0, respectively. The blocked membrane was then incubated with the pre-adsorbed anti-Foxo3a or anti-Foxo3b antiserum (1:1000), or anti-ACTB (β actin) monoclonal antibody (1:500; catalog number: 60008-1-Ig; ProteinTech Group, Inc., IL, USA) in a blocking solution (5% non-fat milk powder in 10 mM PBS) at room temperature for 2 h, washed with PBS for 5 min three times, and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000 dilution, Jackson ImmunoResearch Laboratories, Inc., PE, USA) for 1 h at room temperature. After three 10-min final washes with PBS, the membranes were exposed to a chemiluminescence substrate (ECL; Applygen Technologies Inc., Beijing, China) according to the manufacturer’s instructions.

Quantitative analysis of immunoreactive Foxo3a and Foxo3b levels in ovarian follicular cells

The immunoreactive levels of Foxo3a and Foxo3b in the ovarian follicular cells were analyzed with the Image Pro Plus software (Media Cybernetics, Inc., PA, USA) in a way similar to our previous study (Lu et al. 2014). All the photoimages for analysis were taken under the same conditions. Briefly, the follicular cell layer surrounding an oocyte was defined as the region of interest (ROI). For each ROI, the Foxo3a and Foxo3b expression was shown as DAB staining in the follicular cell layer. Within the follicular cell layer, the sum integrated optical density (IOD) value of DAB staining and area of total follicular cell layer were calculated using the “Count and Measure” tools of Image Pro Plus software. The mean density for Foxo3a or Foxo3b in each ROI, which corresponds to the immunoreactive level, was defined as the sum IOD divided by the sum area of the follicular cell layer. The
measurement for each fish was based on 6 follicles randomly selected from 3 sections at the
interval of about 450 μm, and three fish samples were analyzed for each ovarian development
stage. The data are presented as means ± SEM (n=3).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed to examine the binding of Foxo3 to the cyp19a1a promoter using the
nuclear extracts from COS-7 cells transfected with Foxo3a expression vector pcDNA3.0-Ec
Foxo3a, Foxo3b expression vector pcDNA3.0-Ec Foxo3b, or the empty expression vector
pcDNA3.0. The COS-7 cells were grown in 100-mm dishes, and transfected with 20 μg of
pcDNA3.0-Foxo3a, pcDNA3.0-Foxo3b, and pcDNA3.0, respectively. After 48 h of
transfection, COS-7 cells were washed with PBS, and nuclear proteins were extracted using
Nuclear and Cytoplasmic Extraction Reagents (Beyotime) according to the manufacturer’s
instructions. The nuclear extracts were assayed for protein concentrations with a BCA Protein
Assay Kit (Beyotime), and then kept at -80 °C until use.

The wild-type (W) and mutated (M) oligonucleotide probes for the Foxo binding site of
cyp19a1a promoter were designed based on the sequences between -99/-93 bp. The sequences
of all the sense and antisense oligonucleotides are shown in Supplementary Table 1. The sense
and antisense oligonucleotides are Eccyp19a1a-Foxo3-WT and Eccyp19a1a-Foxo3-WT-C for
the wild-type probe, and Eccyp19a1a-Foxo3-Allmut and Eccyp19a1a-Foxo3-Allmut-C for the
mutated probe, respectively. The annealed oligonucleotides were end-labeled with
[α-32P]dCTP (3000 Ci/mmol) using Exo-free Klenow fragment (New England Biolabs, MA,
USA). Approximately 1.5 ng of labeled probe and 22.5 μg of nuclear extract were incubated
for 20 min at room temperature in a reaction mix (total volume 30 μl) containing 10 mM
HEPES, 5% glycerol (v/v), 1 mM EDTA, 5 mM MgCl$_2$, 0.1 mM DTT, and 15 ng of poly(dI-dC). The reaction mixtures were separated on 5% nondenaturing polyacrylamide gels. The gels were dried, exposed to a Storage Phosphor Screen (Amersham Biosciences, Buckinghamshire, England), and visualized with a Typhoon 8600 Variable Mode Imager (Molecular Dynamics, CA, USA).

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed with an EZ ChIP™ assay kit (Millipore) by following the manufacturer’s protocol with minor modifications for tissues of orange-spotted grouper. The ovary and liver tissues were crushed to powder in liquid nitrogen, and about 1 g of tissue powder was put into 10 ml of PBS buffer containing protease inhibitors (5 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL aprotinine, 5 mg/mL pepstatin), and fixed in 1% (v/v) formaldehyde for 15 min. Then the tissue powder was homogenized in PBS with Dounce homogenizer on ice. The resulted cell suspension was centrifuged, washed twice, and resuspended in cell lysis buffer (100 mM Tris pH8.1, 100 mM NaCl, 0.1% NP-400) containing protease inhibitors to release the nuclei. The released nuclei were pelleted and resuspended in nucleus lysis buffer (1% SDS, 10 mM ethylenediaminetetraacetic acid, 50 mM Tris pH8.1) containing protease inhibitors. Chromatin was sonicated to be 0.2-1 kb in length by using a sonicator (Sonics Model VC130, Sonics and Materials Inc., CT, USA) with a microtip. The sheared chromatins obtained from the ovary or liver equivalent to 60 mg of starting materials were immunoprecipitated according to the manufacturer’s protocol with either 5 μl of anti-Foxo3a or anti-Foxo3b antiserum or 1 μg of specific antibody against RNA polymerase II (as a positive control) or 1 μg of normal mouse IgG (as a negative control) at
4 °C overnight. Immunoprecipitated chromatin (equivalent to about 1.2 mg starting tissues) was then used as a template for PCR amplification of the following three regions of cyp19a1a promoter: -246/+67 bp using primers CYP19A1PF5 and CYP19A1PR2 for the conserved Foxo site, -1024/-687 bp using primers EC19A1-chip-F2 and EC19A1-chip-R4 for the predicted Foxf1 and Foxl2 binding sites, and -2226/-1921 bp using primers EC19A1-chip-F1 EC19A1-chip-R1 for the predicted Foxl1 site. The sequences of the primers were listed in Supplementary Table 1. The PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide.