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Supplementary Materials

Involvement of Sox9a in chondrogenesis and gonadal development in teleost Nile tilapia (*Oreochromis niloticus*)

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Supplementary Materials and Methods

Animals

Nile tilapia (*Oreochromis niloticus*) fish were reared in recirculating, aerated freshwater tanks at 26 °C under a natural photoperiod. All XX progenies were obtained by crossing an XX male, producing sperm after hormone-induced sex reversal, with an XX female. All XY progenies were obtained by crossing a YY supermale with an XX female. Animal experiments were conducted in accordance with the regulations approved by the Institutional Animal Care and Use Committee of Southwest University (No. IACUC-20181015-12).

Establishment of *Sox9a* mutant lines

The genomic and coding sequences of Nile tilapia *Sox9a* (LOC100693400 and XM_005447985.2) were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). CRISPR/Cas9 was performed to mutate *Sox9a* in Nile tilapia, as described previously (Li et al., 2014). A guide RNA (gRNA) target site containing a restriction enzyme site was selected by identifying sequences corresponding to GGN18NGG on the sense or antisense strand of *Sox9a* using ZiFiT (Zinc Finger Targeter) (https://zift.partners.org/zift/Introduction.aspx). Cas9 mRNAs and *Sox9a* gRNAs were co-microinjected into one-cell fertilized eggs at optimal concentrations of 1 000 ng/μL and 500 ng/μL, respectively. In total, 20 embryos were separately collected from the wild-type (WT) and injected groups at 72 h after injection. Genomic DNA extracted from the pooled embryos of each group was subjected to polymerase chain reaction (PCR) using specific primer pairs. The PCR products were then used to screen F0 mutants by sequencing. F1 offspring were produced by crossing females with F0 XY males. The heterozygous *Sox9a* mutant fish with a 7 bp deletion were used to generate homozygous *Sox9a* mutants (*Sox9a*$^{-/-}$). A heteroduplex mobility assay was performed using polyacrylamide gel electrophoresis (PAGE) to screen the mutants, as described previously (Jiang et al., 2016). The PCR primers are listed in Supplementary Table S1. The genetic sex of each fish was determined by genotyping using a sex-linked marker, as described previously (Sun et al., 2014).

Whole-mount *in situ* hybridization

For whole-mount *in situ* hybridization, the cDNA fragment of Nile tilapia *Sox9a* (including 395 bp 5′ untranslated region and 197 bp coding sequence) and cDNA fragment of Nile tilapia *Col2a1a* (including 237 bp coding sequence and 285 bp 3′ untranslated region) were cloned into the pGEM-T Easy vector, respectively. These plasmids were then applied to synthesize digoxygenin (DIG)-labeled probes against *Sox9a* and *Col2a1a* RNAs using a DIG RNA-labeling Mix Kit (Roche, Basel, Switzerland) and T7 RNA polymerase (Promega, Madison, USA). The related primers used are listed in Supplementary Table S1. Whole-mount *in situ* hybridization was carried out using the prepared RNA probes, as described previously (Thisse & Thisse, 2008). Hybridization signals were immunodetected

with an anti-digoxigenin-AP antibody (Roche Mannheim, Germany), then stained using the BCIP/NBT system (Promega, Madison, USA). Images were captured using a Zeiss stereomicroscope (Germany).

Fluorescence *in situ* hybridization

For fluorescence *in situ* hybridization, the Nile tilapia fish were eviscerated at 5, 20, 30, and 60 days after hatching (dah), then fixed overnight in 4% polyformaldehyde (PFA) at 4 °C. All samples were dehydrated, embedded in paraffin, and sectioned at 5 μm. The sections were then deparaffinized, hydrated, and hybridized with DIG-labeled *Sox9a* RNA probes overnight at 60 °C. The sections were subsequently incubated with a horseradish peroxidase (HRP)-conjugated anti-DIG antibody (Roche, Basel, Switzerland), and expression signals were amplified using the TSA™ Plus Fluorescein & Tetramethylrhodamine (TMR) System (PerkinElmer, MA, USA). Finally, the sections were stained with 4′,6′-diamidino-2-phenylindole-dihydrochloride (DAPI; Invitrogen, MA, USA) and confocal images were captured on a Zeiss LSM 880 Laser Scanning Microscope (Germany).

Alcian blue staining

Alcian blue staining was performed to analyze the effects of the *Sox9a* mutation on Nile tilapia chondrogenesis, as described previously (Fujimura & Okada, 2008). In brief, *Sox9a*$^{+/+}$ and *Sox9a*$^{-/-}$ fish at 10 dah were prepared and fixed in phosphate-buffered saline (PBS) containing 4% (wt/v) PFA overnight at room temperature. The fixed fish were gradually dehydrated in 25%, 50%, 70%, and 100% ethanol, then stored at 4 °C until use. The fish were bleached in ethanol containing 5% (v/v) H_2O_2 for 12 h under fluorescent lighting to remove pigmentation. For Alcian blue staining of cartilage, the samples were placed in deionized water and rinsed several times, then transferred into a 20% (v/v) glacial acetic acid solution in ethanol containing 0.02% (wt/v) Alcian blue (Sigma, St. Louis, USA) and incubated overnight at room temperature. The samples were then twice treated (3–6 h per time) with saturated sodium borate solution, before digestion in trypsin solution for approximately 30 min to clear head tissue. The samples were gradually transferred into 30%, 50%, and finally 70% (v/v) glycerol, then observed under a Zeiss stereomicroscope (Germany). Full terms for abbreviations in Figure 1A are as follows: bh, basihyal; bs, basisphenoid; cbs, ceratobranchials; ch, ceratohyal; ch?, presumed ceratohyal; co, scapulocoracoid; ep, ethmoid plate; lon, lamina orbitonasalis; ep, ethmoid plate; mc, Meckel's cartilage; pc, pectoral fin cartilage; pq, palatoquadrate; rc, rostral cartilage; tma, taeniae marginals anterior.

Calcein staining

Calcein staining was used to detect calcium uptake in living animals, as described in previous studies (Du et al., 2001; Fujimura & Okada, 2008). Living *Sox9a*$^{+/+}$ (*n*=3) and *Sox9a*$^{-/-}$ (*n*=3) fish were immersed in 0.2% (wt/v) calcein (Sigma, St. Louis, USA) solution in Petri dishes for 15 min, then rinsed five times in deionized water. The samples were observed under a Leica stereomicroscope (Germany).

Immunofluorescence

The *Sox9a*$^{+/+}$ and *Sox9a*$^{-/-}$ fish were sampled at 5, 8, 12, and 16 dah after removing the intestines. Samples containing the gonads were fixed with 4% PFA for 30 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked in 5% bovine serum albumin (BSA)/PBS for 30 min at room temperature. The samples were then incubated with polyclonal antibodies in 5% BSA/PBS overnight at 4 °C. The polyclonal antibodies against several proteins, including Vasa, Gsdf, and Cyp19a1a, were produced by our laboratory. Alexa Fluor 488- and 594-conjugated secondary antibodies (Thermo Fisher scientific, Waltham, USA) were diluted to 1:1 000 in blocking solution and incubated with the tissue samples for 2 h at 37 °C to detect primary antibodies. The nuclei were stained by DAPI (Invitrogen, MA, USA). Confocal images were captured on a Zeiss LSM 880 Laser Scanning Microscope (Germany).

Germ cell counting

For germ cell counting, the gonads of each *Sox9a*$^{+/+}$ (*n*=5) and *Sox9a*$^{-/-}$ (*n*=5) fish were stained with anti-Vasa antibodies at 8, 12, and 16 dah. Images were acquired using a Zeiss LSM 880 Laser Scanning Microscope (Germany) for counting of germ cells at different time periods.

EdU staining

EdU staining was carried out to detect proliferating cells in the *Sox9a*$^{+/+}$ (*n*=5) and *Sox9a*$^{-/-}$ (*n*=5) fish at 15 dah. Immunolocalization of EdU was performed using a Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, Carlsbad, USA). Germ cells were labeled using anti-Vasa antibodies, followed by DAPI staining. Confocal images were captured on a Zeiss LSM 880 Laser Scanning Microscope (Germany).

Statistical analysis

All data for cell number quantification are presented as mean ± SD. Significant differences between two groups were determined using unpaired two-tailed Student′s *t*-test, with $P<0.05$ accepted as significantly different (* $P<0.05$ versus control).

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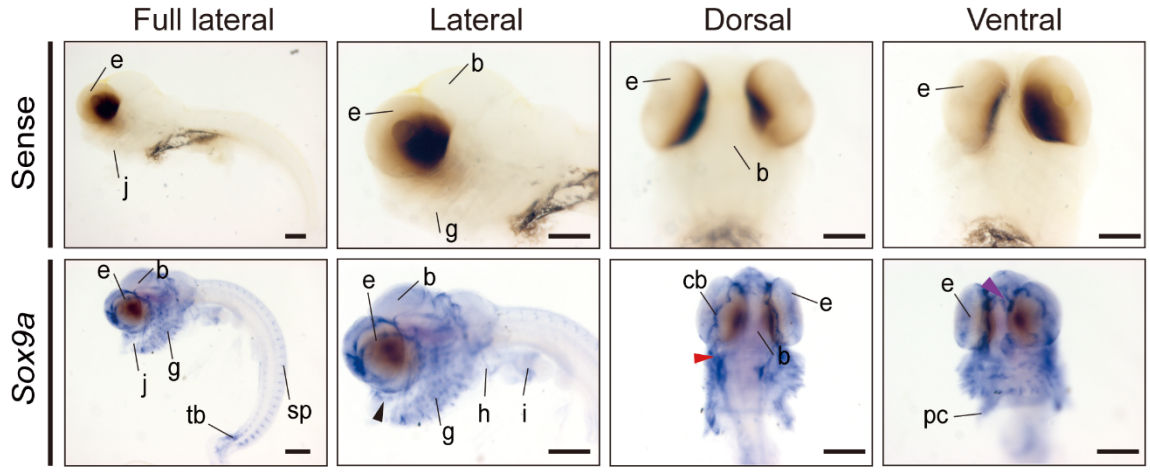
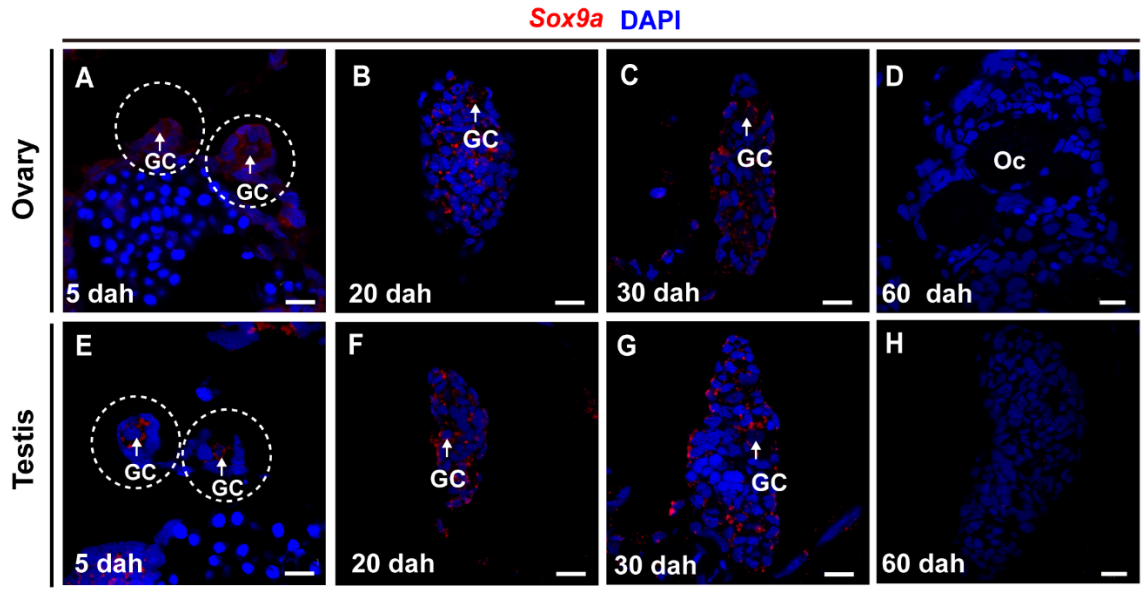
Thisse C, Thisse B. 2008. High-resolution *in situ* hybridization to whole-mount zebrafish embryos. *Nature Protocols*, **3**(1): 59–69.



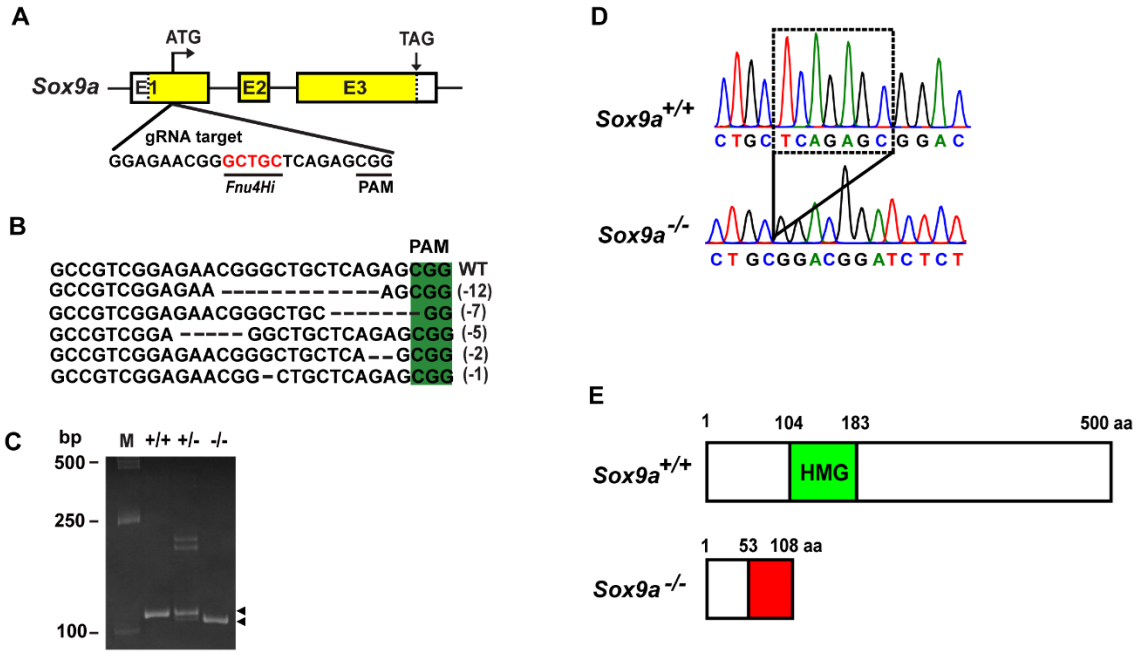
Supplementary Figure S1 Whole-mount *in situ* hybridization analysis of *Sox9a* expression in Nile tilapia at 3 dah

b, brain; cb, cranial bone; e, eye; g, gill; h, heart; i, intestine; j, jaw; pc, pectoral fin cartilage; sp, spinal cartilage; tb, caudal bud cartilage. Black arrowhead, jaw; red arrowhead, skull; purple arrowhead, brain. Dah, days after hatching. Scale bar, 20 μm.



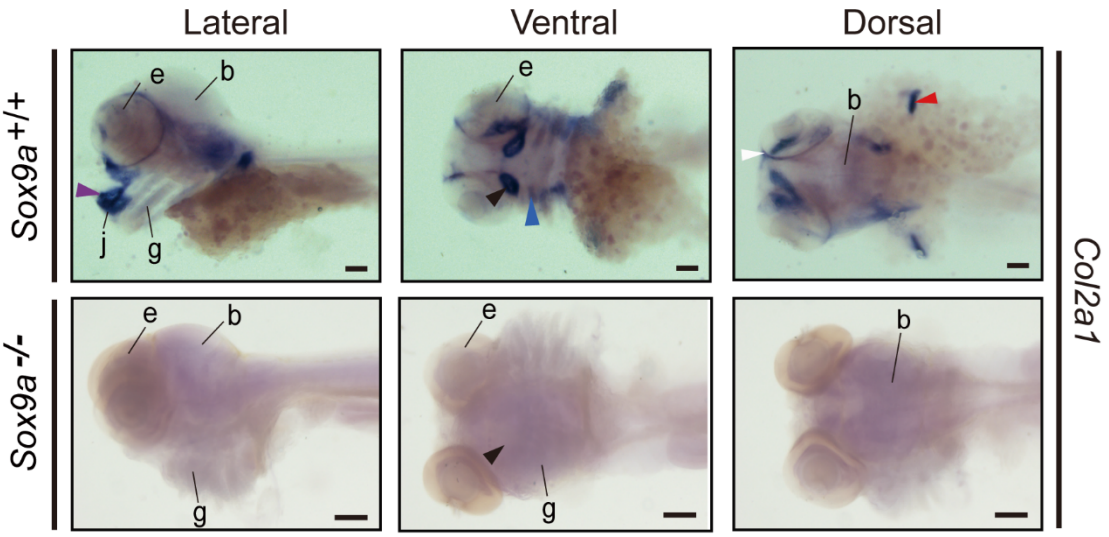
Supplementary Figure S2 *Sox9a* expression in Nile tilapia gonads at different time points

A-D: *Sox9a* expression in ovary of Nile tilapia at 5, 20, 30, and 60 dah. **E-H**: *Sox9a* expression in testis of Nile tilapia at 5, 20, 30, and 60 dah. White arrow indicates germ cell (GC). GC, germ cell; Oc, oocyte; Scale bar, 10 μm.



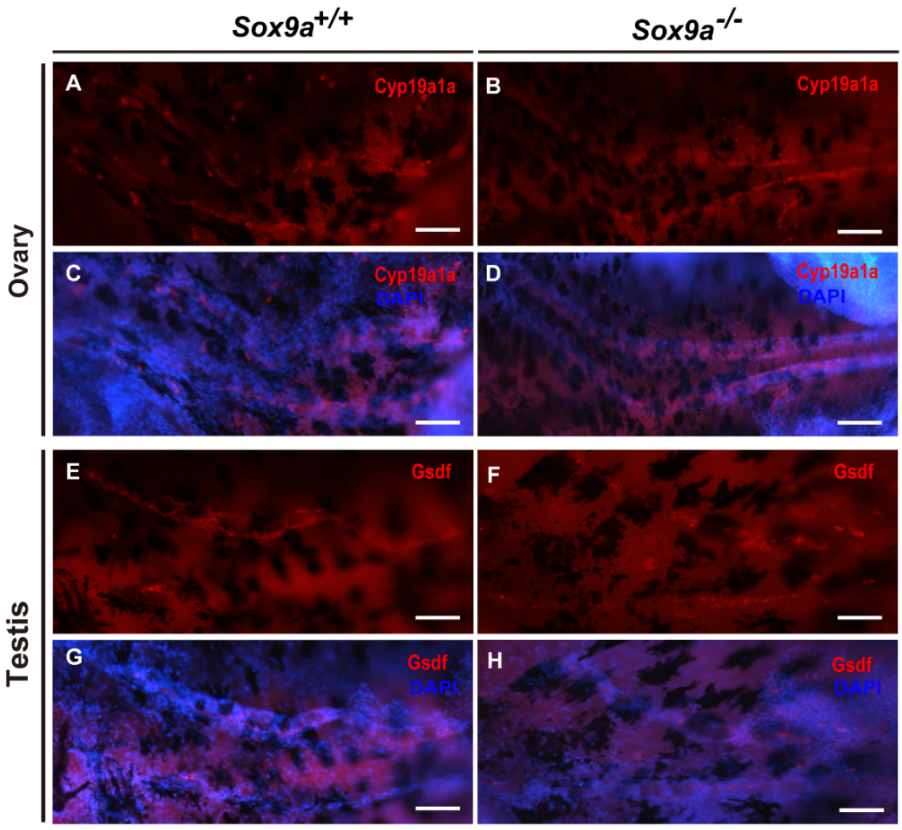
Supplementary Figure S3 Generation of CRISPR/Cas9-mediated Nile tilapia *Sox9a* mutants

A: Schematic of Nile tilapia *Sox9a* gene structure and sgRNA-target site, with one sgRNA target site located on the first exon of the sense strand. **B:** Generation of Nile tilapia *Sox9a* mutant. Mutations at *Sox9a* locus were detected by DNA sequencing. Deletions are represented by dashes, and protospacer adjacent motif (PAM) is indicated in green. Numbers in parentheses represent base loss for each allele. WT, wild-type. **C-D:** Construction of homozygous *Sox9a* mutant. Homozygous mutant was identified by a heteroduplex gel motility assay (C). Further DNA sequencing showed a 7 bp deletion in the first exon of *Sox9a* in mutant fish compared to WT fish (D). **E:** *Sox9a* mutation resulted in premature translation termination. White stars and black arrowheads indicate heterologous and homologous double strands, respectively. *Sox9a*$^{+/+}$, WT; *Sox9a*$^{-/-}$, homozygous mutant.



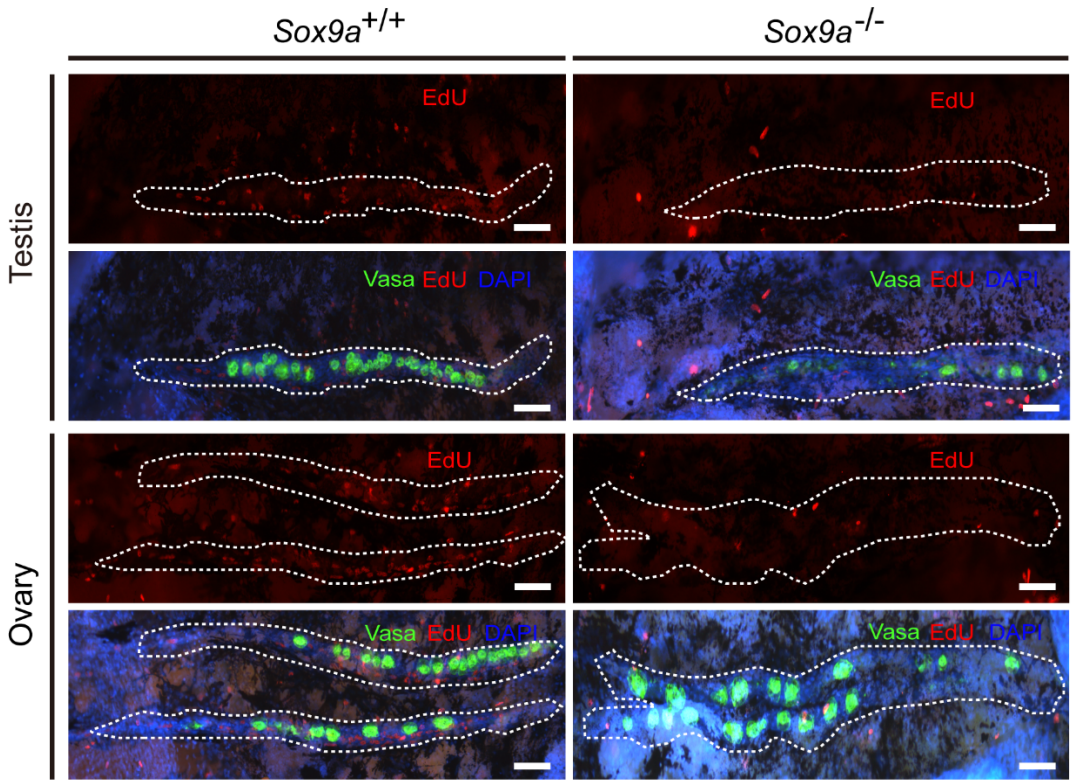
Supplementary Figure S4 Effects of *Sox9a* mutation on *Col2a1a* expression in Nile tilapia cartilage

Purple arrowhead, lower jaw; black arrowhead, mandible; blue arrowhead, gill; white arrowhead, skull; red arrowhead, pectoral fin. b, brain; e, eye; g, gill; j, jaw. Scale bar, 25 μm.



Supplementary Figure S5 *Sox9a* mutation did not affect early sex differentiation in Nile tilapia gonads

Cyp19a1a (A-D) and Gsdf (E-H) expression levels were detected in *Sox9a*$^{+/+}$ and *Sox9a*$^{-/-}$ Nile tilapia gonads at 5 dah by immunofluorescence analysis. Consistent with the pattern found in WT Nile tilapia, Gsdf was expressed in XY gonads of the homozygous *Sox9a* mutants, while Cyp19a1a (aromatase) was expressed in XX gonads of the homozygous *Sox9a* mutants. More than five gonads were examined in each experiment. Nuclei were stained with DAPI. Scale bar, 50 μm.



Supplementary Figure S6 Effects of *Sox9a* mutation on DNA replication in the ovary and testis of Nile tilapia

EdU staining showed that *Sox9a* mutation impaired DNA replication in somatic cells surrounding germ cells in the ovary and testis of Nile tilapia at 15 dah. Scale bar, 50 μm. Germ cells were stained with an anti-Vasa antibody.